The potential of resistant starch to function as a novel infant prebiotic and enhance Zn bioavailability.

Dr Geetha Gopalsamy BSc, BMBS (Hons), FRACP

A thesis submitted for the degree of Doctor of Philosophy

School of Medicine
Faculty of Health Sciences, Flinders University

July 2017

Contents

List of Figures

List of Tables

Abstract

Prior to the current scientific and community interest in the gut microbiota, there was an awareness that adult consumption of resistant starch, a component of dietary starch which is undigested in the small intestine and available for fermentation in the colon, could confer health benefits. More recently it has emerged that the manipulation of the developing gut microbiota during early childhood can have effects on the health of the host that persist into adulthood. These key concepts have converged to form the genesis of this doctoral thesis. Utilising *in vitro* methodology, this thesis examines the capacity of infant faecal inocula to firstly ferment a form of resistant starch, high amylose maize, and then to determine if any such fermentation is associated with microbial changes that might predict a health benefit to the host. If the prebiotic properties of resistant starch are available earlier in the human lifespan, when both the microbiota and the immune system have a greater degree of plasticity, then this might confer novel, durable benefits upon human health. The study presented in Chapters 3 and 4 of this thesis, is unique in many ways: it has the highest number of participants compared to any similar infant *in vitro* fermentation study, it retains a measure of individual variation in fermentation capacity by not pooling participants' faecal inocula and finally, it collects pre-weaning and weaning samples from the same infants in order to demonstrate how the introduction of solids might affect the prebiotic potential of resistant starch. The major aim of Chapter 3 was to determine if the faecal inocula of young infants firstly have the capacity to ferment resistant starch, as determined by an increase in the production of short chain fatty acids and a reduction in pH following 24 hours of *in vitro* incubation. While there was evidence of some fermentation capacity by pre-weaning faecal inocula, the relative increase in SCFA production following incubation of the weaning faecal inocula with the resistant starch suggests that RS intake might be only of clinical significance if provided to infants who have already commenced solid food. In contrast, currently available prebiotics such as fructo-oligosaccharides and galacto-oligosaccharides are administered even to pre-weaning infants. Nevertheless, these results provide the first evidence that resistant starch, in the form of high amylose maize, can be fermented by the faecal inocula of infants who have only recently commenced solids.

Another aim of Chapter 3 was to examine whether there was any difference in the capacity of weaning faecal inocula to ferment a modified starch in comparison to its unmodified counterpart. Sections of the community have expressed concerns regarding the use of modified starches in infant products. This is despite an absence of supporting evidence and the existence of strict government restrictions regarding the degree of chemical modification and the percentage of modified starch that can be added to infant products. The results showed that there was no significant difference in the capacity of the weaning faecal inocula to ferment HAMS in comparison to the commercially available mHAMS. The major aim of Chapter 4 was to use molecular methods, including next generation sequencing (NGS), to characterise the changes to the microbiota

following incubation of the infant faecal inocula with HAMS and a mHAMS. In contrast to the pre-weaning infants, incubation of the weaning faecal inocula with the resistant starches led to dramatic changes in the composition of the microbiota. For example, following incubation with either of the resistant starches, the abundance of Bifidobacteria was higher at 24 hours than at 0 hours, suggesting that both substrates were able to selectively stimulate the growth of this beneficial microbe from human infant microbiota. NGS analysis also demonstrated that in weaning infants, the abundance of Bacteroides significantly increased during fermentation of both HAMS and mHAMS. In weaning infants, fermentation of the resistant starches also increased diversity. Thus in infants who have only recently commenced solids, fermentation of HAMS and mHAMS may indeed have a prebiotic effect. Human trials will be needed to confirm any health-promoting effects. The fermentation of carbohydrates also creates an acidic environment in the large intestine which can facilitate the absorption of divalent cations such as calcium and magnesium. In infants, deficiency in zinc, another divalent cation, is of major significance in the arena of global child health. Thus, in combination with the *in vitro* work already mentioned, this thesis also considers whether the fermentation of high amylose maize might promote the absorption of zinc in a young animal model. To explore such conjecture, a review of the capacity of the large intestine to participate in the absorption of zinc was required. This is presented in Chapter 5 of this thesis. Based on this review, it was discovered that conditions that might reveal a latent capacity for colonic zinc absorption had not been adequately tested. This includes examination of the effect of preceding zinc deficiency on the capacity of the colon to participate in the absorption of zinc. Thus, in Chapter 6, the main aim was to use a weanling rodent model to examine if preceding zinc deficiency affects the capacity of resistant starch to influence zinc bioavailability in a growing mammal. The study revealed that although HAMS increased zinc status, a finding that correlates with previously published studies, there was no apparent effect of preceding zinc status on the capacity of the RS to promote the absorption of zinc. However, in light of other observations also made in Chapter 6, it is apparent that manipulation of host dietary zinc has implications for microbial fermentation activity, and by inference, the composition of the gut microbial community. The effects of dietary zinc on the activity of the microbiota as opposed to the effect on host parameters, have not been well described and the findings of this rodent study bring to light the need for further research into this topic.

Declaration

I declare that the work carried out in this thesis is my own, with the following exceptions as outlined below. I devised and designed both the infant faecal fermentation study and the animal study. I was also responsible for their submissions to the respective ethics committees. For the animal zinc study I manufactured the necessary diets under the guidance of Dr Ying Hu. I was personally responsible for the maintenance and observation of the animals for the duration of the study. I received logistical support from Dr Ying Hu, Dr Jean Winters, and Roshini S during euthanasia of the animals and the collection of relevant biologic samples. The measurement of zinc in the biological samples was performed by SA Pathology. I measured SCFAs in the caecal digesta at CSIRO, Adelaide under the supervision of Ben Scherer.

In relation to the infant fermentation study, I was solely responsible for the recruitment of participants for the study. I received instructions regarding the use of the anaerobic chamber from Michelle Vuaran and Jennifer Giles at CSIRO, Adelaide. I personally performed all faecal collections, fermentation experiments and substrate pre-digestion. I received advice regarding the methodology for the *in vitro* fermentation experiments from Dr Claus Christophersen. I received advice regarding methodology for substrate pre-digestion from Dr Bruce May, CSIRO. I personally performed all SCFA measurements. I also personally performed all molecular experiments, except for the final preparation of the library for deep sequencing. This was performed by Dr Lex Leung at Flinders University. I performed analysis of the sequencing output with the support of Dr Claus Christophersen and an in-house classification pipeline produced by Dr Paul Greenfield at CSIRO.

Prior to submission, I utilised the services of professional editor Dr Robert Muller. Editing was limited to formatting, proofreading and grammatical advice. There was no alteration to the substantive content or conceptual organisation of the thesis.

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed.....Date

Acknowledgment

I would like to thank my primary supervisor, Prof Graeme Young for his astute insight, guidance and encouragement throughout the completion of this thesis. It was a privilege to work under his supervision. To my co-supervisor Dr Claus Christophersen, thank you for your patience, detailed editing and advice. This thesis would not have been possible without your involvement. To my co-supervisor, Dr Ying Hu; I thank you for your friendship and valued assistance. To the extraordinary researchers with whom I worked with at CSIRO, Adelaide and FCIC, Flinders University; thank you for the generous provision of your time and experience. I would also like to thank the NHMRC for awarding me a scholarship.

I dedicate this thesis to my family. To my husband Paul and our sons Erik and Jared; this thesis has required significant sacrifices from all of us. By having to stretch our bonds across the various cities, we have revealed the extraordinary strength of our family. Finally, I am greatly indebted to my parents for their constant support and encouragement.

Abbreviations

ae	Amylose extender mutation
ADE	Acrodermatitis enteropathica
ANOVA	Analysis of Variance
cAMP	Cyclic adenosine monophosphate
CF	Crisp Film
CS	Control Starch
CSIRO	Commonwealth Scientific Research Organisation
DNA	Deoxyribonucleic acid
DS	Degree of Substitution
EAACI	European Academy of Allergy and Clinical Immunology
EE	Environmental Enteropathy
ESPHGAN	European Society for Pediatric Gastroenterology, Hepatology and Nutrition
FAO	Food and Agriculture Organisation of the United Nations
FDA	Food and Drug Administration, USA
FFAR	Free Fatty Acid Receptor
FOS	Fructo-oligosaccharide
FZA	Fractional Zinc Absorption
GBSS	Granule bound starch synthase
GC	Gas Chromatography
GH	Gloycoside Hydrolase
GIT	Gastrointestinal Tract
GOS	Galacto-oligosaccharide
GPCR	G-protein coupled receptors
GPR41	G-protein coupled receptor 41
GPR43	G-protein coupled receptor 43
GRAS	Generally regarded as safe
HAMS	High Amylose Maize Starch
HDAC	Histone Deacetylase Inhibitor
JEFCA	Joint FAO/WHO Expert Committee on Food Additives
mHAMS	Modified High Amylose Maize Starch
NASPGHAN	North American Society for Pediatric Gastroenterology, Hepatology and Nutrition
NCD	Non communicable diseases
NGS	Next Generation Sequencing
ORS	Oral Rehydration Solution

OTU	Operational Taxonomic Unit
PBS	Phosphate buffered saline
PCO	Principle Components Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational multivariate analysis of variance
PHGG	Partially Hydrolysed guar gum
qPCR	Quantitative PCR
RS	Resistant Starch
SBE	Starch binding enzyme
SCFA	Short chain fatty acid
SD	Standard Deviation
SEM	Standard Error of the Mean
SHIME	Simulator of the Human Intestinal microbial Ecosystem
TIM-2	TNO in vitro model of the colon
WAO	World Allergy Organisation
WHO	World Health Organisation

Publications and Presentations

Publications

Gopalsamy, G. L., D. H. Alpers, et al. (2015). The relevance of the colon to zinc nutrition.Nutrients **7**(1): 572-583.

Gopalsamy G, et al. Mo1200 Fermentation of a Modified Resistant Starch by Fecal Bacteria of Young Infants Is Comparable to That of Adults: A Preliminary Report Gastroenterology , Volume 148 , Issue 4 , S-637

In preparation for publication:

Gopalsamy, G, Christophersen, C, Rogers, G, Young, GP (2017) **In vitro evaluation of high amylose maize starch as a novel prebiotic during infancy**. For submission to Journal of Pediatric Nutrition and Gastroenterology.

Invited presentations (each also accepted for poster presentations)

Gopalsamy, G, Australia Gastroenterology Week, Brisbane 2014. Free paper presentation Dietary resistant starch improves bone zinc content in rats regardless of preceding zinc status

Gopalsamy, G, Australia Gastroenterology Week, Brisbane 2015 Free paper presentation. Modified Resistant Starch Maybe a Novel Prebiotic in Infancy

Gopalsamy, G, 5th Science of Nutrition in Medicine Conference 2015 May, Melbourne Fermentation of resistant starch by faecal bacteria of infants.

Chapter 1 Introduction

The developmental origin of many human diseases maybe inextricably linked to early variation in the composition and function of the gut microbiota. The diet of infants and young children has a profound effect on the emerging microbiota, and is thus likely to be a critical parameter in determining the future health of the host. It has been suggested for instance, that the rapid increase in the prevalence of non-communicable diseases (NCDs) in the Western world maybe due to changes in early dietary practices and the resultant perturbation of intestinal microbial colonisation during a critical developmental window (West, Renz et al. 2015). Given the dynamic interaction between gut microbes and the human immune system, such changes will influence the balance between pro-inflammatory and regulatory responses – the fundamental physiological basis of many NCDs (Ismail, Licciardi et al. 2013).

Dysbiosis is defined as a "shift in the balance of the intestinal microbiota to an unbalanced, non-physiological state, resulting in increased growth of harmful bacteria and the decreased growth of protective bacteria" (DeGruttola, Low et al. 2016). In low income countries, dysbiosis of the intestinal microbiome is involved in the pathogenesis of major childhood diseases such as malnutrition and acute childhood diarrhoea (Lawrence, Ana et al. 2015). Globally, these conditions, with their concomitant adverse effects on immune function, result in the majority of deaths in children aged less than five years (Kotloff, Nataro et al. 2013).

Given the above, there may be no better period than in early childhood, when host immune function and the composition of the gut microbiota is being established, to use dietary measures to predictably engineer a gut microbiota that will benefit both short and long term host health. Intervening in adulthood may be less efficacious, as the adult microbiome is a more stable structure which is relatively resilient to short-term dietary interventions (Yatsunenko, Rey et al. 2012). Furthermore, the extent to which the microbiota can influence host parameters, such as the adaptive immune response, may diminish over the course of the human lifetime (Lozupone, Stombaugh et al. 2012). Thus, conducting dietary interventions in early childhood, perhaps even within the first year of life, may result in benefits that extend well beyond childhood to achieve long lasting health outcomes of global importance.

I propose that resistant starch could be one such early dietary intervention. Resistant starch refers to starch that has escaped digestion in the small intestine and is available for fermentation by colonic microbes (Englyst, Trowell et al. 1987). Microbial fermentation refers to the enzymatic decomposition and utilisation of organic material by microbes (Stanbury, Whitaker et al. 1995). The fermentation of high amylose maize starch (HAMS), a form of resistant starch, results in increased microbial production of short chain fatty acids (SCFAs).

These metabolic by-products are critical in mediating many of the beneficial effects of microbes on immune and systemic health (Rios-Covian, Ruas-Madiedo et al. 2016). HAMS may also selectively increase the growth of bacteria, such as bifidobacteria, which are associated with recognised health benefits (Picard, Fioramonti et al. 2005). The administration of HAMS to young infants is a novel proposal. It has been previously espoused that the health benefits of resistant starch would not be available to such young subjects as they may not have the necessary bacteria to ferment such a complex carbohydrate. However, this has never been formally tested. In a clinical trial setting, HAMS in combination with an oral rehydration solution (ORS), has been administered to older children to shorten the duration of acute diarrhoea (Raghupathy, Ramakrishna et al. 2006). As opposed to the current oral rehydration solution (ORS), which is accessible to all ages, there is no recommendation as to the age at which a child could benefit from a novel HAMS-ORS intervention.

Zinc deficiency is a major killer of young children in low income countries and is associated with increased susceptibility to acute diarrhoea. In rodents, resistant starch is effective in promoting zinc bioavailability, possibly through increased absorption of zinc from the large intestine. This thesis will also consider the evidence for the absorption of zinc from the human colon, and will use a young animal model to determine whether preceding zinc status could influence the capacity of resistant starch, in the form of HAMS, to influence zinc absorption.

Chapter 2 Literature Review

2.1 The Gut Microbiota

The human gut microbiota consists of a complex community of microbes which include fungi, viruses, and bacteria. The number of these microorganisms may approximate 100 trillion cells, ten times the number of human cells. Furthermore, the expression of microbial genes outnumbers the expression of human genes by more than 150 times (Ley, Peterson et al. 2006). The composition of the gut microbiota is extraordinarily dense and diverse, containing more than 1,000 different species, many of which cannot be cultured. Although bacteria are present along the entire length of the gastrointestinal tract, their numbers are highest in the large intestine (Canny and McCormick 2008).

Beginning at birth, gut microbes are involved in a formidably diverse range of activities and influence human life at many levels. They are involved in such varied functions as maturation and integrity of the host immune system (Olszak, An et al. 2012), production of essential vitamins and amino acids (Burkholder and McVeigh 1942) and defence against gastrointestinal pathogens (Fukuda, Toh et al. 2011). Gut microbes are also involved in the harvesting, storage and expenditure of energy obtained from the diet (Royall, Wolever et al. 1990). In the adult large intestine, the majority of bacterial species belong to only four phyla, Bacteroides and Firmicutes (which together account for >90% of the species present), as well as Proteobacteria and Actinobacteria (Eckburg, Bik et al. 2005). However, at the genus and species levels there exists much greater diversity (Eckburg, Bik et al. 2005).

As yet there exists no definition of the composition of a “healthy” gut microbiota. This is due in part to the significant inter-individual variation in the human gut microbiota. However, by comparing different compositional backgrounds from diseased patients, it is possible to identify disease phenotypes that might correlate with particular microbial patterns; although it might not always be evident as to whether the dysbiotic gut microbiota is a cause or a consequence of the disease. A reduction in microbial diversity might also be linked to disease states (Sepehri, Kotlowski et al. 2007; Chang, Antonopoulos et al. 2008; Jonkers 2016).

Given the accumulating evidence demonstrating the importance of the gut microbiota in the pathogenesis of disease, it is understandable that strategies to manipulate the gut microbiota, including faecal modification therapy or the intake of pre and probiotics, are being widely explored (Khoruts and Sadowsky 2016). Although each therapeutic modality has a different mode of action, a common goal of these strategies is to augment the microbiota in such a way as to promote a beneficial health outcome.

However, even in health, numerous factors can influence the composition and function of the gut microbiota, including age, genetics and the environment. Such factors present major challenges to the study of the influence of the gut microbiota on host health.

2.1.1 Characterisation of the Gut Microbiota

Recognition of the complexity and variability of the human gut microbiota was only made possible following the advent of DNA-based, culture independent genomic studies (Hiergeist, Gläsner et al. 2015). Older, culture-based techniques to characterise the gut microbiota were limited by the fact that only a small proportion of human gut bacteria is cultivable. It is also now recognised that culture-dependent methods were inherently biased, as micro-organisms can only be cultivated if experimental conditions duplicate their perceived physiological niche (Grice and Segre 2012).

Early culture-independent analysis of the composition of environmental samples utilised the amplification of a distinct cellular component of bacteria 16S ribosomal RNA from extracted DNA, the cloning of these sequences within bacteria and subsequent comparison to a library of known sequences (Ward, Weller et al. 1990). The 16S rRNA gene is a component of the 30S small subunit of prokaryotic ribosomes. It is approximately 1.5 kb in length and is highly conserved between different species of Bacteria and Archaea. In addition to the highly conserved regions, it also contains hypervariable regions that provide species-specific sequences which can be used for bacterial identification (Clarridge 2004).

Initially, a major factor limiting the usefulness of 16S rRNA to characterise the microbial composition of an environmental sample was the expense and time-consuming nature of traditional sequencing methods. However, in 2005, next generation sequencing (NGS) was introduced and the cost and time required to analyse a sample was substantially reduced. Currently, several different platforms for NGS are widely used with the common element being the amplification of single strands of a fragment library and the performance of sequencing reactions on the amplified strands. As opposed to traditional sequencing approaches, there is no requirement for a bacterial cloning step to amplify the genomic fragments and millions to trillions of observations may be made in parallel during a single instrument run. Concurrent advances in the field of bioinformatics now facilitate analysis of the large amounts of data which emerge from the sequencing of such complex ecosystems as the human gut microbiota.

2.2 Diet and the Gut Microbiota

Utilising such analysis, it has emerged that **diet** is a major factor profoundly affecting the composition and function of the gut microbiota (Scott, Gratz et al. 2013). In evolutionary terms, the delegation of part of the digestion and caloric harvest of dietary substrates to resident gut bacteria might have provided a significant advantage to the human host. Due to their genomic plasticity, the gut microbiota would have been able to adjust rapidly to significant day-to-day or season-to-season variation in available food, thus benefiting their human host (Sonnenburg and Sonnenburg 2014).

In adults, children, and weaning infants, the principal dietary substrate for fermentation is dietary fibre. Substrates such as dietary, microbial or host-derived proteins and glycans may also be fermented, but their contribution to overall fermentation is far less (Scott, Gratz et al. 2013).

2.2.1 Dietary Fibre

The Codex Alimentarius Commission is based on cooperation between the FAO (Food & Agriculture Organisation of the United Nations) and the WHO (World Health Organisation. In 2009 the Commission provided a consensus definition regarding the definition of dietary fibre (Lupton, Betteridge et al. 2009). They defined DF as “carbohydrate polymers with ten or more monomeric units which are not hydrolysed by the endogenous enzymes in the small intestine and belong to the following categories:

- Edible carbohydrate polymers occurring in food as consumed
- carbohydrate polymers which have been obtained from raw materials by physical, enzymatic, or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities, and
- synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities. (Lupton, Betteridge et al. 2009)”

Within the boundaries of this definition, naturally occurring dietary fibres maybe subclassified according to their chemical composition as follows; non-starch polysaccharides, resistant oligosaccharides (including cellulose, fructo-oligosaccharides, and inulin), resistant starch, and lignin and plant substrates such as phytate and tannins. The effects of dietary fibre on gut health and microbiota composition have been studied using a variety of research methods including *in vitro*, animal, and human studies. Dietary fibres modify microbial composition by both stimulating bacteria that may directly feed on them, and by facilitating the growth of cross-feeding bacteria that may depend on the products of metabolism by the primary degraders (Conlon and Bird 2015).

A diet high in fibre also provides a mechanism for “competitive exclusion” whereby the expansion of commensal bacteria which can utilise dietary fibre may limit the opportunity for more pathogenic bacteria to gain a foothold (Lee 2013). It has been previously thought that the two main phyla Bacteroidetes and Firmicutes are predominantly involved in utilizing fibre due to their large range of carbohydrate utilizing enzymes. However, as will be discussed, other groups of bacteria including those from the *Bifidobacterium* genus, may also be adept at carbohydrate and in particular starch utilisation. The major health effects of dietary fibre fermentation are mediated through the production of short chain fatty acids (SCFAs) (Cook and Sellin 1998).

2.2.1.1 Short chain fatty acids

Short chain fatty acids are comprised of only 1-6 carbon atoms and are formed predominantly in the large intestine through the microbial fermentation of dietary fibre (Gijs, Van Eunen et al. 2013). The three major SCFAs, acetate, propionate, and butyrate are produced in considerable quantities within the colonic lumen, reaching total concentrations of around 100mM. The individual concentrations tend to occur in a ratio or 3:1:1 with acetate being the most prevalent, followed by propionate and butyrate (den Besten, van Eunen et al. 2013; Gijs, Van Eunen et al. 2013). Although the total concentration of SCFAs decreases from proximal to distal segments of the colon, the ratio of the three major SCFAs remains approximately the same (Cummings, Pomare et al. 1987). The lesser SCFAs include formic, isobutyric, valeric, isovaleric and caproic acids, and these are found in much lower concentrations within the colonic lumen. The pathways for the production of the three major SCFAs from the microbial fermentation of dietary fibre into monosaccharides are illustrated in Fig 2-1. Bacterial crossfeeding can also lead to the generation of SCFAs; for example, the production of butyrate from acetate,(see also Fig 2-1). This particular method of generating SCFAs is known as metabolic cross-feeding. Another form of bacterial cross-feeding is substrate cross feeding, whereby bacteria that are not capable of utilising the initial complex non-digested carbohydrate, are able to utilise the breakdown products produced by fermentation of the substrate by other microbes (Rios-Covian, Ruas-Madiedo et al. 2016).

Fig 2-1 has been removed due to copyright restrictions

Figure 2-1 Schematic representation of the formation of the three major short chain fatty acids through the microbial fermentation of dietary fibre and cross feeding. (Rios-Covian, Ruas-Madiedo et al. 2016).

Over 95% of SCFAs are absorbed from the colon (Topping and Clifton 2001). Absorption of SCFAs can also promote the absorption of $Na^{\pm}$, a finding that will be explored further in discussions regarding the role of SCFA in colonic salvage of water during episodes of acute diarrhoea (Binder and Mehta 1989). In a healthy adult, 5-10% of total energy requirements can also be obtained from the absorption of SCFAs. Such a mechanism facilitates the harvesting of energy from material that

may escape digestion in the small intestine. In lowland gorillas, one of the closest living relatives of modern humans, 57% of their energy requirements may be obtained from SCFAs (Popovich, Jenkins et al. 1997).

The potential contribution that absorption of these acids might make to the energy needs of a growing child has not been quantified. Theoretically, if small intestinal absorptive function is compromised, i.e., as might occur in environmental enteropathy or short bowel syndrome, the contribution of absorbed SCFAs to total energy requirements could be even greater.

Due to differences in their chemical structure, the physiological effects of the different SCFAs vary. For example, butyrate, while also being the primary energy source for the colonic epithelium (Roediger 1982; Donohoe, Garge et al. 2011), is a histone deacetylase (HDAC) inhibitor – a property which has led to interest into the potential of butyrate to protect against colorectal cancer (McIntyre, Gibson et al. 1993; Medina, Edmonds et al. 1997; Burn , Bishop et al. 2008). As almost all butyrate is consumed by the colonocyte, it is mainly acetate and propionate which enter into the portal circulation. Propionate is primarily used by the liver in gluconeogenesis and as such it is not found in significant quantities in the systemic circulation. Acetate is the dominant SCFA to be found in plasma (Pouteau, Meirim et al. 2001).

The varied effects of SCFAs on immune function and gut homeostasis have become a major focus of research. It is now recognised that SCFAs may promote the integrity of the gut barrier, through stabilisation of the protective mucus layer (Willemsen, Koetsier et al. 2003) and the enhanced expression of proteins which reduce intestinal permeability (Peng, He et al. 2007). The integrity of the gut epithelial barrier is critical for human health. If the barrier is compromised, translocation of potential antigens and bacteria-derived products, such as lipopolysaccharides, from the lumen into the systemic circulation can occur. Such movement may initiate an inflammatory response, disrupt gastrointestinal homeostasis and compromise normal pathways of immunogenic tolerance (Bischoff, Barbara et al. 2014). Ultimately, these events may be etiologic factors in the development of autoimmune diseases and even metabolic phenotypes such as obesity.

SCFA may also enact their effects directly on the host immune system by binding to various G protein coupled receptors (GPCRs). These include FFAR2 (free fatty acid receptor 2, also known as GPR43) and FFAR3 (GPR41) (Brown, Goldsworthy et al. 2003; Le Poul, Loison et al. 2003). These recently discovered GPCRs may be found in various cell types, including gut epithelial cells, adipocytes, and immune cells such as macrophages or dendritic cells (Layden, Angueira et al. 2013). The different SCFAs differ in how avidly they bind to FFAR2 and FFAR3. For example, FFAR2 appears to selectively bind to acetate and propionate, while FFAR3 may selectively bind to propionate and butyrate (Layden, Angueira et al. 2013). Activation of FFAR2 may both improve insulin resistance and regulate the inflammatory response (Maslowski, Vieira et al. 2009; Ichimura,

Hasegawa et al. 2014). Regulatory T cells (Tregs) are critical for suppressing the potentially deleterious activities of Th cells, and it is now known that Tregs express FFAR2 receptors (Smith, Howitt et al. 2013). Thus by binding to these receptors, SCFAs modulate the Treg cell response and can exert a potential anti-inflammatory effect (Smith, Howitt et al. 2013).

2.3 The Gut Microbiota in Early Life

The manipulation of the microbiota in early life may have far greater health consequences than if such manipulation occurs in later years when the system is more resistant to perturbation (Gibson, Crofts et al. 2015). Studies conducted in both humans and animals suggest that transient disturbances to the microbiome in early life can have durable effects on host metabolic and immune function; even if the composition of the microbiome recovers (Roduit, Scholtens et al. 2009; Rautava, Luoto et al. 2012; Russell, Gold et al. 2012; Gibson, Crofts et al. 2015). An intervention deemed to be of “benefit” during this window period, might confer protection against the development of future disease and conversely interventions which deplete the community of critical members or compromise the functional capacity of the microbiome during this crucial period, may increase the risk of developing future disease.

For example, in a study involving 236 children aged from two to five years, those who had received macrolide antibiotics in early life (and thus suffered a disruption to their gut microbiota), had an increased risk of asthma and antibiotic associated obesity (Korpela, Salonen et al. 2016). The largest part of the human immune system is contained within the intestinal mucosa (Mowat and Agace 2014). Exposure to external antigens, derived from such sources as food and environmental microorganisms, requires the cells of the mucosal immune system to discriminate constantly between harmless and pathogenic antigens. Within this context, the commensal microbiota are essential for the development of a normal immune system, particularly in relation to adaptive immunity and the programming of T cell differentiation, including the response of antigen specific regulatory T cells (Treg). Furthermore, it is recognised that there is a finite period during which the developing immune system is amenable to microbial instruction.

Given the complex interactions between the gut epithelium, The host immune system, and the gut microbiota, prebiotic and probiotic interventions conducted early in human life, could reduce the prevalence of future immune mediated disease (Tang and Lodge 2016). However, before considering which interventions, if performed in early childhood, may benefit host health, it is necessary to have a further understanding of how the infant gut microbiota differs from that of an adult, and what the important factors are which already impact upon its development.

2.4 Establishing the human gut microbiota

The human microbiota is commonly thought to be established at birth. However, this historical assumption that the human foetus is microbiologically sterile may not be accurate as there is evidence suggesting that the foetus is exposed to bacteria *in utero* (Mueller, Bakacs et al. 2015). Regardless of this potential for intrauterine exposure, during vaginal delivery, bacterial colonisation of the infant with maternal vaginal and intestinal microbiota is an important source of colonizing microbes for the infant (Thum, Cookson et al. 2012; Endo, Tang et al. 2015). In caesarean delivery the initial colonizing organisms differ, representing environmental bacteria from non maternal sources (Gronlund, Lehtonen et al. 1999).

With vaginal delivery, the initial pioneer organisms that colonize the infant's intestine are facultative anaerobic bacteria, such as Escherichia coli, Staphylococcus, and Streptococcus. Over the first few days of life, these bacteria consume all the oxygen in the environment, enabling strict anaerobes such as Bacteroides and Bifidobacterium to colonize and then multiply. In comparison to adults, the infant's gut is highly dynamic in nature, with significant interindividual variation (Faith, Guruge et al. 2013). This relative instability may make the infant gut microbiome more susceptible to modulation or disruption by environmental factors. It may also be a factor in explaining the apparent increased susceptibility of young children to gastrointestinal infections.

Alterations in 'normal' patterns of colonization can occur due to differences in delivery mode, the level of prematurity, antibiotic exposure and whether the infant is formula fed and/or breastfed (Bergstrom, Skov et al. 2014). The influence of these external factors on the development of the infant gut microbiota is illustrated in Fig 2-2.

Figure 2-2 has been removed due to copyright restrictions

Figure 2-2 The impact of external factors on the intestinal microbiota of the infant. Green arrows demonstrate beneficial modification; red arrows show modification considered negative for healthy development (Matamoros, Gras-Leguen et al.).

2.4.1 Breast-feeding versus formula-feeding

Exclusive breast-feeding has a profound influence on the composition of the infant microbiota. A prominent component of breast milk is human milk oligosaccharide, which is solely produced in lactating mammary glands. Human breast milk contains more than 130 different forms of human milk oligosaccharides (HMOs). HMOs survive in the low pH of the gut and resist digestion by pancreatic and brush border enzymes. Consequently, they are available as a nutrient source for colonic bacteria. In the large intestine, they are fermented to SCFAs and lactic acids, reducing the pH of the local environment. The ability of bifidobacteria to utilise human milk oligosaccharides has enabled these species to be among the most abundant colonizers of the breast-fed infant gut.

There exist demonstrable differences between the faecal microbiota of exclusively breastfed and formula-fed infants; although there are some inconsistencies in these findings (Guaraldi and Salvatori 2012). A few studies have found that compared to breast-fed infants, bifidobacteria are not found in comparable amounts in the gut microbiota of formula-fed infants.

However, other studies refute these results and claim that even in formula fed infants, bifidobacteria can still predominate (Scholtens, Oozeer et al. 2012). In addition to differences in molecular methodologies, these varied findings may also reflect the increasing use of exogenous oligosaccharides, which are bifidogenic, and probiotics such as *Bifidobacterium longum,* in commercial infant formula (Vandenplas, Zakharova et al. 2015).

2.4.1.1 Bifidobacteria and the infant gut

It is likely that bifidobacteria have co-evolved with their host over millions of years, to express critical health effects on the host, particularly during the infantile period. The relative abundance of bifidobacteria in the adult colon is much lower than in infants, representing only around 4% of total faecal microbes (Ventura, Turroni et al. 2015). The genus Bifidobacterium belongs to the family of Bifidobacteriaceae, belonging to the order of Bifidobacteriales and the phylum Actinobacteria*,* one of the dominant phyla of the kingdom Bacteria. Bifidobacteria are gram-positive saccharolytic bacteria, with the vast majority being strict anaerobes (Bondue and Delcenserie 2015). Bifidobacteria have a range of beneficial effects on the host and in particular, infant health. *Bifidobacterium spp* can synthesize several B group vitamins, including folate, biotin, thiamine and B12. Particularly in the case of folate, it is known that the extracellular release of the vitamin from the bacteria into the colonic lumen, can improve the vitamin status of the host (Pompei, Cordisco et al. 2007). *Bifidobacterium spp* may also be involved in the strengthening of the intestinal epithelial barrier (Hsieh, Osaka et al. 2015). The potential adverse effects of disruption to the intestinal epithelial barrier were discussed earlier (Pastorelli, De Salvo et al. 2013). The production of organic acids such as lactate and acetate by bifidobacteria also serves to lower intestinal pH and thereby inhibit the growth of more pathogenic organisms (Kailasapathy and Chin 2000). Using an animal model, Fukuda et al. demonstrated that the provision of bifidobacteria could protect mice against death induced by *E Coli 0157: H7* (Fukuda, Toh et al. 2011)*.* The authors speculated that although the effect might be attributed to the increased production of acetate by the bifidobacteria, it might also have been due to inhibition of the translocation of the E Coli O157: H7 Shiga toxin from the gut lumen to the systemic circulation (Fukuda, Toh et al. 2011).

2.4.2 The Introduction of Solids

The optimal duration of breastfeeding was reviewed by the Cochrane collaboration in 2012 (Kramer and Kakuma 2012). The authors found that infants who are exclusively breastfed for six months, experience less morbidity from gastrointestinal infection than those who are partially

breastfed for three or four months. Studies conducted in Finland and Australia, found no evidence of a significant reduction in risk of atopic eczema, asthma or other atopic outcomes in infants who were exclusively breast fed for 6 months(Kajosaari 1994).

More recently it has been suggested that the introduction of potentially allergenic solid foods before 6 months of age maybe associated with a decreased risk of developing food allergy (Wei-Liang Tan, Valerio et al.). Whilst acknowledging that the current NHMRC guidelines, published in 2012, recommend that infants are exclusively breastfed until 6 months of age, there is no consensus in the literature regarding the age of complementary food introduction as a means of allergy prevention. In Australia only 15 % of infants are exclusively breast fed to 6 months of age. (Australian Institute of Health and welfare. 2010 Australian National Infant Feeding Survey: www.aihw.gov.au/WorkArea/DownloadAsset)

The transition from breast or formula-feeding to a solid diet is accompanied by rapid and dramatic changes in the composition and metabolic function of the infant microbiome (Fallani, Amarri et al. 2011). The exposure to novel dietary polysaccharides encourages new species of bacteria to emerge which are capable of utilising these novel sources of energy. Although worldwide weaning practices vary considerably, globally, most children are first introduced to solid foods at approximately 4-6 months of age (Fewtrell, Morgan et al. 2007). Weaning is defined as the transition from milk feeding to solid foods and does not imply the cessation of breast-milk.

Unfortunately, there are very few studies addressing the effects of the early complementary diet (solid food) of infants after six months of age on later gut microbial composition and immune function. Stark et al. demonstrated that while bifidobacterial counts may remain stable throughout the first year of life, the introduction of solids in exclusively breast-fed babies is associated with increased colonisation by other groups of bacteria including Bacteroides and Clostridium (Stark and Lee 1982; George, Nord et al. 1996). More recently, utilising modern, culture-independent techniques, Fallani et al examined the faecal microbiota composition of 605 European infants using samples taken approximately four weeks after the introduction of first solids (Fallani, Amarri et al. 2011). They compared the results to samples taken from the same infants prior to weaning. The study confirmed that although the genus Bifidobacterium was still dominant (36.5% of total detectable bacteria), there was a significant increase in species of Clostridium and Bacteroides.

Following the introduction of solid foods, the phylogenetic diversity of the microbiome continues to increase with age. This is not only due to an expanding range of dietary substrates which are available to the microbiota, but it may also be a result of changes to host immune parameters (Koenig, Spor et al. 2011). Between the ages of two and three, a functionally stable microbiota that is generally similar in composition to that of adults, is established. However, some studies have

suggested that even at the age of five, the gut microbiota can still be distinguished from that of adults in terms of composition and diversity (Ringel-Kulka, Cheng et al. 2013). Despite this uncertainty regarding the exact age at which the adult-like microbiota emerges, it is generally accepted that once this core microbiota develops and stabilises, it is less susceptible to perturbation (Yatsunenko et al 2012).

In light of this resilience against long term diet induced changes in the microbiota of adults,the composition of the weaning diet may have far reaching consequences for host physiology (Saha and Reimer 2014). It is certainly possible that complex carbohydrates at the time of weaning may enrich for microbiota with increased fermentative capacity, which in turn could have long term benefits for the host (Conlon and Bird 2015).

As mentioned previously, weaning practices differ considerably across the world. In many Western countries, including Australia, iron-fortified rice cereal is often recommended as the first weaning food (NHMRC 2013). However, across many parts of the world, young infants are introduced to much more complex carbohydrates as their first solid food (Sajilata, Singhal et al. 2002). Interestingly, it is not until weaning that major geographical differences in microbial composition emerge (De Filippo, Cavalieri et al. 2010). This likely reflects the conserved effect of breast-milk on the microbiota across varied maternal dietary patterns and diverse genetic and cultural backgrounds.

In a landmark study by De Fillipo et al, microbial composition and function were compared between healthy rural African children and European children (De Filippo, Cavalieri et al. 2010). In this study, the African group demonstrated an altered ratio of two dominant phyla of the human microbiota, with increased representation of Bacteroides relative to Firmicutes. They also had greater microbial diversity and a larger increase in faecal SCFAs. This correlated with a reported increase in the intake of dietary fibre amongst the African cohort in comparison to the European cohort. The study concluded that a much higher fibre content in the African diet resulted in a distinct microbiota with a higher prevalence of bacteria primed for carbohydrate fermentation. However, what cannot be deduced from this study is whether the divergence in microbial profile and metabolic function was related to the initial age at which complex fibres were introduced into the diet. Indeed, the age at which the microbiota of a young child first develops the capacity to ferment complex carbohydrates has not been clearly defined. Furthermore, it is likely that such a capacity is substrate-dependent.

It is possible that unless fibre exposure occurs early in life, there may be an irreversible loss of species that cannot be recovered upon increased dietary fibre intake in later life (Sonnenburg and Sonnenburg 2014). Indeed, animal studies support the assertion that the age of introduction of dietary fibres may have a profound influence on microbial diversity, microbial function and host

physiology in later life (Young, Roy et al. 2012; Saha and Reimer 2014). As mentioned earlier, **resistant starch** is a form of dietary fibre. In the following few sections I will review the basic structure and characteristics of resistant starch, its fermentation and the evidence regarding its potential beneficial health effects.

2.5 Resistant Starch

Resistant starch (RS) represents a diverse range of indigestible starch-based dietary carbohydrates that are capable of being fermented by colonic microbiota (Sajilata, Singhal et al. 2006). A variety of beneficial health effects have been prescribed to RS in adults, including chemoprevention in colorectal cancer, control of intestinal transit and bowel habits, a reduction in postprandial glycaemia, increased insulin sensitivity and stimulation of growth and function of the gut microbiota (Fuentes-Zaragoza, Riquelme-Navarrete et al. 2010).

Starch is the most abundant storage reserve for carbohydrates in plants. It is used by plants as a source of energy during periods of regrowth and dormancy and is found in many different plant organs, including roots, fruits, seeds and tubers (Jobling 2004). Due to its versatility and useful physicochemical properties, starch is also used in a number of different industrial applications,

Starch is formed from two types of glucose polymers – amylose and amylopectin, (see Fig 2-3). These molecules are organised into a semi-crystalline granular structure. Amylose is composed of linear chains of glucose united through α(1-4) linkages. Amylopectin is a much larger molecule, and is heavily branched with basic repeating units of α(1-4) linked glucose with branches of α(1-6) linked glucose. Branching occurs irregularly in the starch molecule.

Fig 2-3 has been removed due to copyright restrictions

Figure 2-3 Chemical structure of amylose and amylopectin (Buleon, Colonna et al. 1998)

The amylose-amylopectin ratio of starch may vary considerably. Normal maize starch may contain approximately 20-30% amylose, while the amylose content of a selectively bred high amylose maize starch may be around 70%. As will be discussed later, this ratio of amylose – amylopectin can markedly change the extent and rate at which a starch molecule can be digested by amylase, an enzyme involved in starch digestion.

2.5.1 Starch digestion in humans.

Starch digestion and absorption is a multifactorial process (Mathers and Wolever 2009). Starch is broken down enzymatically by a process of hydrolysis in the presence of the enzyme amylase, resulting in the release of glucose and fragments of starch such as maltose, maltotriose, and

dextrins. Amylases are a class of enzymes that are capable of digesting the glycosidic linkages in the starch molecule. In humans, salivary α-amylase released during chewing inititates the initial digestion process. It had been held that due to the highly acidic environment of the stomach, salivary amylase will be rapidly deactivated prior to dietary products entering the proximal small intestine (Singh, Dartois et al. 2010).

However, there is *in vitro* evidence to suggest that salivary alpha amylase may be protected from acid digestion by starch molecules and its hydrolysis products (Rosenblum, Irwin et al. 1988). Despite this, it is generally agreed that pancreatic α-amylase performs the majority of dietary starch hydrolysis in humans (Butterworth, Warren et al. 2011). Pancreatic α-amylase, is secreted by the pancreas and is released into the small intestine through the pancreatic duct. It hydrolyzes amylose and amylopectin at random to maltose and dextrins. In humans another type of enzyme is also involved in breaking down starch molecules, mucosal α-glucosidase. These enzymes are predominantly involved in the conversion of post α–amylase dextrins to glucose, but they may also act synergistically with α amylase and participate in the initial stage of starch hydrolysis (Dhital, Lin et al. 2013).

The starch granule size is quite large in comparison to the α amylase molecule (Butterworth, Warren et al. 2011). Although there are many potential binding sites of the enzyme within the intact starch granule, the complete breakdown of the starch is a relatively slow process (Butterworth, Warren et al. 2011). Other factors that may affect the rate of starch breakdown in the small intestine include the gastric emptying rate, intestinal motility, and absorption and metabolism of starch degradation products.

2.5.2 Forms of resistant starch

It was initially thought that all starch was completely digested and absorbed in the small intestine. However, in the 1980s it was recognised that there was a component of starch that may be resistant to digestion which behaves like a dietary fibre (Englyst, Wiggins et al. 1982). The mechanism of its resistance to digestion in the small intestine segregates various forms of resistant starch into 5 categories. RS1, RS2, RS3, RS4 and RS5, (see Table 2-1).

Table 2-1 Types of Resistant Starches (Champ 2004; Hasjim, Ai et al. 2013)

Designation	Description	Example
RS1	Physically inaccessible starch	Coarsely ground or whole kernel grains, legumes
RS2	Granular starch	High amylose maize starch, raw potato, raw banana starch
RS3	Retrograded starch	Cooked and cooled starchy foods
RS4	Chemically modified starches	Cross linked starch, acetylated starches
RS5	Amylose lipid complex	Stearic acid-complexed high amylose starch

2.5.3 High amylose maize starch

Starch obtained from high amylose maize varieties contains a type 2 resistant starch. In starch with a high amylopectin component, due to its more branched and less dense structure, amylase can access the starch structure more readily and is thus more susceptible to hydrolysis. High amylose starch (HAMS) on the other hand, has a smaller number of glucose units and is less branched. This results in a more linear shape which is tightly packed and less accessible to the amylase enzymes, making it more resistant to digestion (Brown, Macnamara et al. 2000). In human ileostomy subjects, approximately 30-50% of dietary HAMS is undigested in the upper gastrointestinal tract and would thus be available as a substrate for colonic bacteria in those with intact alimentary tracts (Muir, Birkett et al. 1995; Vonk, Hagedoorn et al. 2000; Clarke, Bird et al. 2007).

The heating of RS2 granules in water, results in their swelling (gelatinization), and thus, the loss of their resistance to digestion. Gelatinization of other forms of RS2, such as those found in potatoes and green bananas, occurs at below 70^{o}C, whereas gelatinization of HAMS granules does not occur fully until temperatures of 154-171^{0}C are reached (Wang, Conway et al. 1999b). This property of HAMS, along with its small particle size, bland flavour, and white appearance, enables it to be readily incorporated into foods designed for human consumption (Ashwar, Gani et al. 2016).

2.5.3.1 Production of high amylose maize starch

Before considering the potential use of HAMS in infant nutrition, some attention must be given to how starch synthesis is manipulated to achieve high amylose cultivars. Three enzymes are responsible for the synthesis of starch from ADP–glucose; starch synthases, starch branching

enzymes and starch debranching enzymes (Zeeman, Kossmann et al. 2010). Starch synthases catalyse the addition of glucose units from ADP-glucose onto the non-reducing ends of amylose or amylopectin chains. Multiple isoforms of starch synthase exist in storage organs. Amylose synthesis is primarily achieved through the action of a single synthase - granule bound starch synthase (GBSS)-the only synthase to be found entirely within the granule (Tetlow and Emes 2011). Mutants lacking GBSS produce amylose free starch, also known as waxy maize. The other starch synthase isoforms contribute to amylopectin formation by elongating chains of different lengths.

Starch branching enzymes produce the α(1-6) branches in starch by cleaving the α(1-4) linkage and then joining the reducing end of the severed fragment to the same or an adjacent chain, producing an α(1-6) linkage (Tetlow and Emes 2011). In maize, there exist at least three isoforms of the starch branching enzyme (SBEIa, SBEII, and SBEIIb), with each isoform differing in chain length transfer pattern and substrate preference (Yao, Thompson et al. 2004).

Loss of SBEIIb, produces the amylose extender (ae-) mutation, resulting in a significant reduction in starch synthesis and proportionately higher levels of amylose than amylopectin (Jobling 2004). Traditional plant-breeding techniques can further increase the relative amount of amylose in maize cultivars carrying mutations in the *ae* gene. The fact that HAMS is not obtained through genetic modification is of particular importance if it is to be approved, or indeed accepted, for use in infant products.

Currently, the majority of starch for use in food and industrial use is obtained from corn (maize) starch. High amylose maize hybrid varieties have a lower yield of starch than dent corn (maize), the variety most commonly used for industrial processes, animal feed and the manufacturing of processed food (McWhirter and Dunn 1986). One reason for this is that the *ae* allele not only affects the amylose:amylopetin ratio, but it also has an effect on the size of the kernel and starch content (McWhirter and Dunn 1986) . This could have cost and supply implications if HAMS is to have a greater role in human nutrition.

2.5.4 Resistant starch as a substrate for gut microbes

As mentioned, resistant starch enters the large intestine where it is available for fermentation by the resident colonic bacteria. The degradation of resistant starch, and indeed all complex carbohydrates in the large bowel, is a cooperative process. It involves the initial degradation of the polymer structure into sugar monomers, glycolysis and production of SCFA and other organic acids, hydrogen consumption during methanogenesis and sulphate reduction (Gijs, Van Eunen et al. 2013) .

2.5.4.1 Amylolytic bacteria

Microbial degradation of starch entails diverse enzymes with different specificities and modes of action. The number and type of amylolytic bacteria capable of digesting a starch may vary considerably depending on the structure and composition of the starch (Birkett, Mathers et al. 2000). It is acknowledged that the capacity to degrade amylose, as opposed to amylopectin, appears to be restricted to far fewer species of bacteria (Wang, Conway et al 1999). Using culture dependent techniques, Wang et al screened 38 human colonic bacteria strains by measuring the clear zone on PY medium agar plates supplemented with both high amylose and high amylopectin starch (Wang, Conway et al. 1999b). The authors found that although several strains of bacteria had amylase activity, only *Bifidobacterium* spp and *Clostridium butyricum* could utilize the high amylose starch granules (Wang, Conway et al. 1999b).

Using an anaerobic fermentor system, Leitch et al (2007), demonstrated that only four species of bacteria from human faecal inoculum, could colonize HAMS, *Eubacterium rectale, Ruminococcus bromii,* and two species of Bifidobacterium – *Bifidobacterium adolescentis* and *Bifidobacterium breve* (Leitch, Walker et al. 2007). It has been speculated that adhesion of the bacteria to the starch molecule may be required for its utilization. Such binding may be very strain and substrate dependent. It has also been proposed that *R.bromii* may be a keystone species in starch digestion (Ze, Duncan et al. 2012). Ze studied the capacity of four dominant amylolytic bacteria to degrade and utilise high amylose maize starch and determined that in comparison to *E.rectale* and *B. Thetaiotomicron*; *R.bromii* and *B.adolescentis* had a similar capacity to degrade RS2.

However, when the researchers then examined the interactions between the four chosen amylolytic strains, they found that when co-incubation with *R.bromii* was performed, there was a significant increase in the utilization of the RS2 compared with monocultures of each strain. This led to the conclusion that *R bromii* was a “keystone” species in high amylose maize starch digestion. However, the authors accepted that more extensive investigations are required before it can be concluded that *R bromii* is essential for RS2 degradatation and utilisation. It would certainly be remarkable if the degradation of a particular starch molecule was dependent on the presence of a single species of bacteria, particularly as functional redundancy appears to be an intrinsic property of the gut ecosystem (Moya and Ferrer 2016). It had also previously been thought that members of the phylum Bacteroides were the most extensive users of polysaccharides in the human gut; however, it has now emerged that bifidobacteria may have a selective advantage in the utilization of starch and starch hydrolysates (Liu, Ren et al. 2015). In 1986, Macfarlane found that of 120 amylolytic colonies randomly selected from faecal samples of six human subjects, 58% were identified as *Bifidobacterium* spp (Macfarlane and Englyst 1986), 18% were identified as Bacteroides spp, and another 10% were identified as *Fusobacterium* and *Butyrovibrio* (Macfarlane and Englyst 1986).

With the advent of more advanced molecular techniques, and by comparing the glycoside hydrolyase (GH) profiles of bifidobacteria with other representative members of the microbiome and through constructing predicted metabolic pathways, it appears that starch and starch hydrolysates are quite favourable carbon sources for bifidobacteria (Liu, Ren et al. 2015). Indeed, Liu and colleagues determined that glycoside hydrolysates participating in the degradation of starch and starch hydrolysates were more abundant in bifidobacteria than in other intestinal bacterial strains. (Liu, Ren et al. 2015).

It was also determined that a complete starch pathway for degrading starch and starch hydrolysates can exist in approximately 25 bifidobacterial genomes (Liu, Ren et al. 2015), suggesting that amylolytic capacity is not limited to only a few species of bifidobacterium. Duranti et al recently also demonstrated that *B.adolescentis*, can thrive on starch as its sole carbon and energy source (Duranti, Turroni et al. 2014). The question as to why bifidobacteria may be so adept at starch utilisation requires some consideration. In infants, the lack of chewing ability, the immature pancreatic amylase activity, and the relative immaturity of the gut may allow a large amount of starch to escape digestion from the upper gut, thereby arriving in an essentially intact form in the infant gut. Bifidobacteria, as mentioned earlier represents a significant portion of the bacteria in the infant's colon. The high repertoire of starch-utilising enzymes expressed by bifidobacteria may ensure that any starch entering the large intestine can be fermented to release important metabolites, such as SCFAs, which can influence immune system development and both colonic and systemic health. By having a wide repertoire of sugar and starch utilization enzymes, bifidobacteria can thus rapidly adjust to a diet initially dominated by human milk oligosaccharides (in breast-milk) to one that is more characterised by plant-based carbohydrates (weaning foods). The primary amylolytic bacteria that can degrade starch are not the only ones that can benefit from its fermentation. Extensive cross-feeding between the microbiota dramatically increases the actual number of bacterial species that are affected by starch fermentation.

2.5.5 Modified starches

Type 4 resistant starches are starches that have been chemically modified to resist digestion and absorption. Chemically modified starches were originally developed for their favourable technical properties. For example, acetylated starch, a form of chemically modified starch, is widely used in the food industry functioning as binding, stabilizing thickening and texturing agents in a variety of commercial food products (Han, Liu et al. 2012). These starches are most commonly produced through esterification of the native starch with either acetic anhydride or vinyl acetate. Despite their widespread use, many national and international food authorities have stipulated that the degree of acetylation in the final product should not exceed 2.5%, corresponding to a maximum degree of substitution of 0.1 (JEFCA 1982a; Han, Liu et al. 2012; FCC 2014).

It was eventually recognised that esterification of starch with acetic acid, or indeed, other short chain fatty acids such as proprionic or butyric acid, could provide a mechanism to deliver SCFAs directly to the large bowel. Not only does esterification reduce the digestibility of the starch in the small intestine, thus increasing available substrate for fermentation, but it also appears that the esterified acid can be liberated into the colonic lumen through the action of microbial esterases and lipases (Morita, Kasaoka et al. 2005).

Once cleavage of the coupled SCFA occurs, the now accessible remaining starch molecule is likely to be fermented in the same way as if it had not been initially modified. The selection of which acid is esterified to the starch backbone can influence fermentation. When acetic acid, a shorter SCFA, is esterified to the starch backbone, the added moiety is unable to lie parallel to the gylosyl units (Bajka, Topping et al. 2006; Lim, Barnes et al. 2014). This leads to a loss of structure within the crystalline domain, resulting in a more open structure which is more accessible to bacterial colonisation and hydrolytic enzymes (Lim, Barnes et al. 2014). In contrast, butyric acid is a longer molecule and thus butylated starch is packaged differently to its acetylated starch counterpart, influencing the nature of its fermentation (Lim, Barnes et al. 2014). The degree of substitution can affect the activity of the microbial esterases through such mechanisms as steric obstruction and a reduction in the solubility of the starch molecule. *In vivo* studies conducted both in animals and humans confirm that the dietary intake of acetylated high amylose maize starch is indeed associated with increased concentrations of the corresponding coupled SCFA in the large bowel of participants (Annison, Illman et al. 2003; Bajka, Topping et al. 2006; Clarke, Bird et al. 2007).

Even at low levels of acetylation there may be advantages in terms of resistance to retrogradation, solubility and the prolonging of storage time (Han, Liu et al. 2012). Acetylated starches conforming to government regulations are currently added to a variety foods, including infant food products, as thickening, binding, stabilising and texturing agents. Crispfilm, manufactured by Ingredion in the USA, is one such widely available commercial product.

2.5.6 The in vivo effect of resistant starch intake on the microbiota

2.5.6.1 Animal studies

In a rodent study utilising only culture based methods to assess changes to the microbiota, the provision of 5 months of RS1 and RS2 (derived from potato starch) was found to stimulate the growth of bifidobacteria in both faecal and caecal samples (Kleessen, Stoof et al. 1997). Only the RS2 also increased the colony counts of Lactobacilli,Streptococci and Enterobacteriacea. Using 16S rRNA gene amplicon sequencing methodology, Tachon et al (2013) found that mice that were fed high amylose maize starch (RS2) were colonized by higher levels of Bacteroidetes and Bifidobacterium (Tachon, Zhou et al. 2013). These findings correlate with those made by Young et al, following 28 days of feeding high amylose maize starch to weanling rats (Young, Roy et al.

2012). In this latter study, the authors noted that although their finding of an increase in Bifidobacterium and Bacteroidetes in response to HAMS intake had been reported previously, their results suggested a more dramatic response. They considered whether their protocol of modifying the microbiota at an earlier age in the animal's lifespan could have had a greater effect on the composition of the microbial community.

As an animal model, pigs have several advantages over rodents in regards to researching the effects of diet on the modulation of the human microbiota. They are equivalent in size to humans, are omnivorous, and their digestive physiology and intestinal microbial community share greater similarity with us than other non-primate animal models (Heinritz, Mosenthin et al. 2013). In a 12 week feeding experiment conducted in growing pigs, a type 3 resistant starch derived from retrogradation of tapioca starch, led to a significant increase in Prevotella and the Ruminococcus genus, which include *R.bromii* (Umu, Frank et al. 2015). This latter finding is in keeping with the *in vitro* studies which suggest that *Ruminococcus* is particularly adept at RS utilisation (Fuentes-Zaragoza, Sanchez-Zapata et al. 2011). Bifidobacterium, which is thought to make only a small contribution to the pig's microbiota, was not detected in any of the faeces, irrespective of diet.

2.5.6.2 Human studies

There have been several *in vivo* studies examining the effect of RS consumption on the gut microbiota of humans. Following several weeks of a diet high in type 2 resistant starch, it was found that there was a significant increase in the abundance of *R.bromii*, *F prausnitzii and E.rectale* (Abell, Cooke et al. 2008) in the faeces of human volunteers. Similar findings were revealed in a controlled 10 week dietary intervention with an RS3 in 14 overweight men, where it was found that when compared to a control diet, the RS intervention again stimulated the growth of *R.bromii* and *E.rectale* (Walker, Ince et al. 2011). In only one of the 14 subjects was a significant increase in Bifidobacteria noted with the RS intervention.

More recently, Venkataraman et al provided 20 healthy young adults with an RS2 derived from potato starch and found significant inter-individual variability in faecal SCFA concentrations (Venkataraman, Sieber et al. 2016). Patients (n=11 of 20) who had a significant increase in faecal butyrate concentrations following RS intake were also noted to have an increase in the relative abundance of RS degrading organisms. For example, in eight of the cases, there was a significant increase in the abundance of *B.adolescentis,* and in three individuals who demonstrated evidence of fermentation but in whom *B.adolescentis* sequences did not increase, there was an increase in the abundance of *R bromii*. Six of the 20 volunteers had low concentrations of butyrate both before and during consumption of the resistant starch and, within this group, there was no increase in the abundance of RS degrading organisms, suggesting that the microbiota of these individuals were unable to utilise the substrate. Indeed, several studies have noted that following a particular dietary fibre intervention, including RS interventions, there can be significant inter-individual variation in

fermentation capacity and subsequent compositional shifts in the gut microbiota.(David, Maurice et al. 2014; Conlon and Bird 2015; Wu, Tremaroli et al. 2015). This is likely to be a reflection of crucial individual differences in baseline microbial composition and function, including variations in the presence of antagonistic microbes or the absence of synergistic microbes (Venkataraman, Sieber et al. 2016). This inc

The chemical structure of the RS may also have an effect on its accessibility to different colonic bacteria and thus, its selective effect on gut bacteria composition (Birkett, Mathers et al. 2000). In a placebo-controlled double blind crossover trial involving 10 human volunteers, it was found that an RS4, but not an RS2, significantly increased faecal counts of both Actinobacteria and Bacteroidetes (Martinez, Kim et al. 2010). The RS2 arm of the study again noted an increase in proportions of *R.bromii* and *E.rectale* (Martinez, Kim et al. 2010).

Similarly, Abell et al found that in comparison to standard high amylose maize starch, the administration of butyrlated high amylose maize starch (RS4) (DS=.25) to rodents was associated with differing effects on the colonic microbiota (Abell, Cooke et al. 2008). The degree and rate of degradation of acylated starch and its effect on gut microbial composition may also depend on which particular SCFA is esterified to the starch backbone. Several bifidobacteria strains have been shown to be capable of degrading acylated starches (Lim, Barnes et al 2013).

2.6 Assessment of the fermentation of dietary substrates in infants

The initial composition of the microbiota has a profound influence on the compositional changes and metabolic activities that occur with fermentation of a substrate (Rios-Covian, Ruas-Madiedo et al. 2016). As was discussed earlier, the composition of the infant and adult gut microbiota differ significantly and, as such, the microbial changes associated with the fermentation of a resistant starch in an adult cannot be readily extrapolated to the infant. Indeed, it cannot be assumed that the infant microbiota will even have the capacity to ferment the resistant starch.

The study of substrate digestion and fermentation in the human digestive system is an expensive and difficult task (Coles, Moughan et al. 2005). *In vivo*, the gut microbial community is a dynamic ecosystem that exhibits a multitude of interspecies and host interactions. The colonic microbiota also receive a variety of host-derived endogenous compounds and anti-nutritional factors which may interact with the fermentation of diet derived substrates (Coles, Moughan et al. 2005). Despite the potentially superior biological significance conferred by studying fermentation *in vivo,* such methods also pose difficulties. The relative inaccessibility of the proximal colon and portal vein necessitates that the measurement of *in vivo* fermentation parameters requires highly invasive procedures. Compliance, volunteer drop-outs and the difficulty in controlling the background diet of study participants are other important considerations. In infants, a unique consideration is the

increased awareness that manipulation of the microbiota during early childhood may have major consequences on adult health, some of which may yet be unknown.

Animal models are unencumbered by many of the restraints faced by human studies, and provide a method to study the *in vivo* interactions between fermentable substrates, gut microbiota and host physiology. Using animals, the variability of external factors such as diet and genetics can be restricted and more invasive sampling can be undertaken. Various animal models have also been developed to better represent the human gut microbiota and its capacity for manipulation. These include conventionally raised (native gut microbiota), germ free (no bacteria present), gnotobiotic (germ free animals colonized with select species), or human flora associated (animals colonized with flora from human faecal suspensions) (Fritz, Desai et al. 2013). However, animal studies also have several limitations. In addition to the fact that they can be expensive and time consuming to conduct, there exist fundamental differences in physiology between humans and animal models that raise understandable questions regarding the relevance of animal findings to human health (Shanks, Greek et al. 2009).

2.6.1 In vitro models of fermentation

In vitro models have been developed as an alternative method to study microbial interactions with a test substrate. There is no one model that is suitable for all scientific questions and, despite many advances, all current *in vitro* models can only confer a representation of what occurs *in vivo*. Models range from simple *in vitro* batch culture methods to more complex continuous culture systems that more closely mimic the human gastrointestinal system (Edwards, Gibson et al. 1996; Coles, Moughan et al. 2005). Whether it consists of a single or multiple vessels, most *in vitro* systems are inoculated with faecal samples and operate under anaerobic conditions. Advantages of *in vitro* models include their ability to create a highly controlled environment, as well as providing an opportunity to screen and compare a number of potential pre or probiotics before performing *in vivo* studies (Payne, Zihler et al. 2012).

The choice of *in vitro* fermentation method depends on cost, the technical expertise available, and the research question being considered. However, the choice of which system is used to model fermentation should not detract from the importance of other experimental factors such as appropriate donor selection, handling of faecal material from donor to reactor, storage, reactivation, and methods to cultivate or analyse members of the gut microbiota. A few studies have confirmed agreement between shifts in gut microbiota composition *in vitro* with actual human feeding studies, underlying the utility of *in vitro* fermentation methodology in predicting fermentation events in the human (Bourquin, Titgemeyer et al. 1996; Daniel, Wisker et al. 1997). For example, the bifidogenic effects of fructo-oligosaccharides and inulin that have been observed in human clinical trials have also been reproduced using *in vitro* fermentation models (Van den Abbeele, Gerard et al. 2011).

Unfortunately the opportunity to compare results across different *in vitro* fermentation studies is limited. This is due in part to there being no standard method, even for simple batch fermentation methods, in regards to duration of the incubation, collection and preparation of the inoculum, composition of the fermentation media, pH of the incubation and analysis of the metabolic and microbial changes. In one study, Kim et al using a variety of molecular methods, found dramatic differences in faecal bacterial community following *in vitro* fermentation, depending on the concentration of carbohydrate in the medium (Kim, Kim et al. 2011). They found that a low concentration carbohydrate medium provides the best growth conditions to conduct *in vitro* studies that are representative of the human intestinal microbiota (Gareau, Wine et al. 2010).

Static faecal batch fermentation involves a closed system (sealed) maintained under anaerobic conditions to allow for growth of faecal communities (Coles, Moughan et al. 2005). There is no addition or removal of material once inoculation of the fermentation medium is performed. Static faecal batch ferments are limited to short term studies, due to both the reduction of substrate availability over time and the accumulation of potentially toxic metabolites which may influence accurate representation of bacterial composition and fermentation profile (Williams, Walton et al. 2015). Static batch ferments typically do not control pH, a factor that can have an influence on both the survival and function of various microbes (Payne, Zihler et al. 2012). However, as an initial assessment of the ability of a faecal microbial community to metabolize a particular substrate, it is considered an efficient, high throughput method (Coles, Moughan et al. 2005).

The continuous culture model of fermentation has characteristics which are closer to the *in vivo* environment (Venema and van den Abbeele 2013; Williams, Walton et al. 2015). It provides for a constant flow of substrate through the system under anaerobic, pH, and temperature controlled conditions. It has the capacity to continuously provide fresh growth medium whilst concurrently removing spent culture medium, waste, and toxic by-products from the system (Williams, Walton et al. 2015). The complexity of the chemostat may vary considerably, with some utilising a simple, single-stage model while others provide representation of the various segments of the GIT by including multiple vessels, each with unique pH conditions (Payne, Zihler et al. 2012). Unlike the batch fermentation method, chemostats can achieve a steady state in relation to human faecal communities, allowing them to be cultured over a much longer time frame (Payne, Zihler et al. 2012). The contents can also be utilized for multiple studies, allowing for more accurate comparison between substrates.

The SHIME (Simulator of the Human Intestinal Microbial Ecosystem) system (Ghent University Prodigest, Ghent, Belgium) has three compartments which represent the different segments of the colon (Molly, Vande Woestyne et al. 1993). The pH in each of the three compartments differs, reflecting *in vivo* conditions. The overall residence time of these three vessels is approximately 72 hours.

Inoculation of the system with faecal microbiota is followed by a two-week stabilisation period during which the faecal microbes adapt to the different *in vitro* conditions. TIM-2 is another dynamic *in vitro* model of the colon consisting of four compartments which express peristaltic-like activity. Unlike SHIME, TIM-2 predominantly seeks to represent fermentation activity only within the proximal colon (TNO, Netherlands). It also uses a novel dialysis membrane to simulate uptake of microbial metabolites by the body, thus preventing accumulation of metabolites which might otherwise inhibit microbial activity (Williams, Walton et al. 2015). Fermentation experiments are usually run over a 24 to 72 hour period.

A particular concern with continuous fermentors is that they may suffer issues with low bacterial cell count within the fermentation system (Cinquin, Le Blay et al. 2006). To account for this, the initial inoculation of such systems usually requires a large amount of initial faecal material. The limited amount of faecal material available from young infants can be an issue when attempting to model fermentation within the infant colon using *in vitro* methodology. In order to circumvent this, some researchers have developed methods to immobilise the initial faecal inocula in 1-2mm polysaccharide gel beads, prior to its use in continuous *in vitro* fermentation systems (Cinquin, Le Blay et al. 2004). Using such an immobilisation method within an infant colonic fermentation model, it was found that the community composition and numbers of bacterial cells in the chemostat could achieve comparable levels to the initial faecal inocula (Cinquin, Le Blay et al. 2004).

The complexity of intestinal simulators continues to develop rapidly. Models now exist that attempt to combine host cellular information with *in vitro* digestive systems, potentially allowing evaluation of host microbiota interactions such as cytokine production, barrier function, absorption, and transport (Williams, Walton et al. 2015). The choice of method utilised for a particular study will depend on cost, the technical expertise available and the research question being considered. For the purposes of this thesis, a static anaerobic batch fermentation model has been chosen. A major reason for this choice, as opposed to a pH controlled continuous system, is the limited quantity of faeces available from young infants. The decision to retain a measure of individual variation by not using pooled faecal specimens further contributes to the minimal quantity of stool available for use in the *in vitro* fermentation experiments. If the quantity of faeces available was not a limiting factor, the use of more complex multistage continuous flow models would still be problematic due to their limited availability and the high costs associated with their development.

2.6.2 Pre-digestion methods

The preparation of the substrate used for *in vitro* fermentation experiments will also affect the extent to which the results agree with an *in vivo* setting (Paeschke and Aimutis 2010). RS preparations used for *in vitro* fermentation studies may contain some digestible starch and as such, it should be subjected to a process of *in vitro* digestion prior to use in such experiments.

However, there can be significant heterogeneity between studies regarding predigestion methodology and indeed whether it is actually performed or not. In adult ileostomy participants, there exists the opportunity to use starch isolated from ileal content as a substrate for further *in vitro* fermentation studies. However, due to the relative rarity of infant ileostomy patients, it is not feasible to employ such a method for infant fermentation studies.

Although there is significant variation in the specific methodology for pre-digestion among *in vitro* fermentation experiments, there are certain common physiological events which need to be simulated. *In vivo*, the oral phase initiates the digestion process. Not only does this involve the mechanical breakdown of food, it also initiates the hydrolysis of dietary starch through the action of salivary α-amylase. For the purposes of replicating the digestion of young infants whose primary intake would be liquid or mashed/pureed food, inclusion of the chewing step is not required. The next phase of digestion is the gastric phase and does require imitation within *in vitro* models of digestion. When a food bolus is presented to the stomach, digestion of protein is achieved through the actions of pepsin within a highly acidic environment. The final step of the pre-digestion method is to replicate the digestive activity of the small intestine. This involves raising the pH to mimic more closely the pH of the small bowel, followed by incubation with exogenous α -amylase and α-amyloglucosidase. A final ethanol precipitation step excludes monosaccharides and disaccharides, oligosaccharides and fructans.

2.6.3 Preparation of faecal samples

Limited access to luminal gut microbiota in healthy participants has resulted in the majority of *in vitro* fermentation studies using faecal material as the initial inoculum. However, this relatively easily accessible source of microbiota, only represents the luminal distal colonic microbiota. There is significant variation in the composition of the microbiota along the gut, both longitudinally from proximal to distal segments, and latitudinally between the lumen and the mucosa (Gerritsen, Smidt et al. 2011)

The composition and function of human faecal inoculum for use in *in vitro* studies may be affected by variations in storage and preparation. Several animal studies have suggested that freezing faeces may damage and disrupt the bacterial cell membrane, resulting in both the release of intracellular contents as well as the loss of certain members of the microbial community (Murray, McMullin et al. 2012). Gram negative bacteria may be particularly vulnerable to the freeze thaw cycle (Nassos, King et al. 1988) However, major adverse effects were not observed during the preparation of human frozen faecal inocula by Rose et al (Rose, Venema et al. 2010). Although a direct comparison of microbial composition between frozen and fresh inoculum was not the main purpose of the study, the authors demonstrated that in faecal specimens stored for 44 weeks at -80^{0}C, the composition of the microbiota and the number of viable cells did not significantly differ

between frozen and fresh inoculates. However, the study did not examine how the preparation of the faecal inoculum prior to freezing might affect preservation of the sample. When bacteria are subject to freeze and thaw cycles, the extent of damage depends on several parameters including the rate of freezing, the rate of thawing, storage temperature and the inclusion of any medium which might act as a cryoprotective agent .

Glycerol can function as a cryoprotective agent (McKain, Genc et al. 2013). Using the TNO dynamic *in vitro* colon model (TIM-2), four different methods to prepare and store human faecal inocula for *in vitro* fermentation studies were studied and the composition and activity of the microbiota between the treatment groups was compared to results from fresh faecal specimens (Aguirre, Eck et al. 2015). The treatment groups differed according to the presence or absence of either glycerol and/or dialysate solution in the faecal specimen prior to freezing. The study concluded that the storage method which approximated most closely to results from fresh faecal specimens, required suspension of faecal inoculum in a dialysate solution containing glycerol, followed by snap-freezing in liquid nitrogen prior to storage at -80^{0}C. The use of fresh faeces for *in vitro* fermentation studies is not always practical, particularly in infant studies, due in part to the unpredictability of infants regarding the frequency of stool passage. For the purposes of my doctoral thesis, I have elected to use stored frozen faecal inocula for my batch *in vitro* fermentation experiments.

2.7 Dietary Starch in Infants

In order to explore the potential for HAMS to benefit infant health, there must be an understanding of the capacity of such young children to hydrolyse and ferment dietary starch. It is well recognised that pancreatic amylase activity in infants significantly lags behind that of adults. For the first three months of life, pancreatic amylase activity in duodenal fluids is very low, and it is not until a child is approximately six years of age, that pancreatic activity achieves the levels of an adult (O'Donnell and Miller 1980).

Salivary amylase activity achieves that of an adult much earlier, within only a few months of life, however, in the absence of mastication, it is unclear as to how much it may contribute to starch digestion during early weaning (Lebenthal and Lee 1980). There is also evidence that an amylase gene is expressed in the lactating mammary gland of humans and other mammals, with alpha amylase being present and active in human breast-milk (Butterworth, Warren et al. 2011). The contribution of breast-milk amylase activity to hydrolysis of dietary starch is unclear. Human milk does not contain starch, but rather, it contains non-starch oligosaccharides that are formed from galactose (Smilowitz, Lebrilla et al. 2014); and α-amylase does not act upon these oligo-saccharides (Butterworth, Warren et al. 2011; Flint, Scott et al. 2012).

In many communities, nursing mothers chew solid food materials containing starch i.e. roots and tubers, and then pass this pre-masticated food to their infants to supplement their breast-milk diet (Aggett 2010). Whether this may also serve to prime the colonic bacteria to deal with more complex carbohydrates that will appear following the introduction of solid food, can only be hypothesised. Given the relative immaturity of α-amylase activity in young infants, the potential for significant amounts of dietary starch to reach the colon is significant.

2.7.1 Infants and starch digestion – in vivo results

Only a few *in vivo* studies have examined the hydrolysis and fermentation of starch in young infants and, as they were conducted prior to the advent of molecular methods, there is a paucity of data regarding the effect of such early intake on the ontogeny of the colonic microbiota. Vize et al examined the digestibility of various starches (potato, tapioca, corn, wheat and rice) given to 1-3 month old infants. By measuring the faecal content of lactic acid, glucose, dextrins, and starch, the amount of starch digested was quantified (De Vizia, Ciccimarra et al. 1975) .It was found that up to ½ a cup of cooked wheat, tapioca, corn, rice, or potato starch, given daily, was almost completely absorbed. In an earlier study, Shulman et al demonstrated starch utilization by 16 healthy 1-month-old infants by tracing the appearance in breath CO_2 of carbon derived from a starch based cereal and, comparing this to values obtained following the ingestion of glucose controls (Shulman, Wong et al. 1983). The authors found that the extent to which the cereal was oxidised was comparable to the glucose controls, as indicated by similar amounts of the doses being oxidised to CO2 (Shulman, Wong et al. 1983). Stools from four infants were analyzed for the quantity of carbon that originated from the cereal. In two of these samples, cereal carbon was detectable but represented a relatively small percentage of the ingested load (3.7% and 13.1%) (Shulman, Wong et al. 1983).

In contrast to these early findings suggesting adequate digestion of starch during infancy, a later study published in 1994 found a significant proportion of dietary starch in the faeces of infants and young children aged less than three years (Verity and Edwards 1994). These results raised concerns about the true extent of starch hydrolysis in early childhood, and led to uncertainty regarding the capacity of the infant colonic microbiota to ferment any undigested starch (Edwards and Parrett 2003).

2.7.2 In vitro assessment of the fermentation of complex carbohydrates by infant faecal inocula

There is a need for evidence based recommendations regarding the intake of dietary fibre, including resistant starch, during weaning and, in general, during the first year of life. Historically there have been concerns that the increased intake of dietary fibre during this vulnerable period in

human development may lead to mineral imbalances, faecal energy and fluid losses, and growth restriction. In the case of resistant starch, there has also been a concern that the microbiota of the younger child may not have acquired the required complexity of bacterial species to ferment such a complex carbohydrate as starch.

More recently, there has been a greater reliance on results from *in vitro* fermentation experiments to examine the capacity of infant faecal inocula to ferment starch. This may reflect the increased cost of clinical studies, a shift in ethical thresholds and a need to accumulate more safety data before substrates are directly fed to young infants. Parrett et al published an early study using *in vitro* batch fermentation to test the fermentation capacity of both breast-fed and formula-fed infants for simple and complex carbohydrates in pre and weaning healthy infants (Parrett, Edwards et al. 1997). The complex carbohydrate chosen for this study was a soybean polysaccharide. The results were compared to those from adult faecal cultures and it was found that, in the infant faecal cultures, very little SCFA was produced following incubation with the soybean polysaccharide, suggesting the absence of fermentation.

Given the substantial differences in microbial colonisation between infants and adults, and the likely lack of prior dietary exposure to complex carbohydrates such as guar gum or soybean polysaccharide, such a result may not be unexpected as there would be no selection pressure for bacteria with the capacity to ferment such substrates (Parrett, Edwards et al 1997).

Also utilising *in vitro* batch fermentation methods, Scheiwiller et al investigated the ability of infant colonic microbiota to ferment resistant starch – in this case a type 3 RS. They found that, prior to weaning, the colonic microbiota of infants was unable to degrade the RS. However, the faecal inocula of their weaning infants did demonstrate the capacity to ferment the type 3 resistant starch.

Crucially, the weaning infants in Scheiwiller's study were older and had likely been consuming solids for a longer duration of time than the weaning infants in Parrett's earlier study. Despite this, when parameters of fermentation, including substrate degradation were compared to results from adult inocula, they concluded that the microbiota of adults was still better adapted to fermentation of the test substrate (Scheiwiller, Arrigoni et al. 2006).

Conversely, Christian et al using pooled faecal inocula from healthy infants (7-10 months), toddlers (16-21 months), and adults, and incubating them with waxy corn starch (high amylopectin), found that fermentation of certain starches in young children may actually be more efficient than in adults (Christian, Edwards et al. 2003). There are several study related differences that may account for the disparity in conclusions between this and the earlier studies by Parrett et al, 2003 and Schweiller et al, 2006. The infants in Christian's study were well established on solid foods prior to being included in the study and there was a difference in the substrates tested.

The concentration of corn starch in the infants background diet was likely greater than the concentration of the substrates used in Parrett's and Schweiller's studies. With proportionately more corn starch then passing undigested into the colon, this may have precipitated adaptive changes in bacterial flora that could then result in greater fermentation of the corn starch (Christian, Edwards et al. 2003). If the *in vitro* studies could be replicated in vivo, the authors suggest that fermentation of corn starch could lead to significant salvage of energy from the colon (Christian, Edwards et al. 2003).

2.7.3 Resistant starch in weanling animals

There have been very few *in vivo* studies investigating the effects of dietary intervention with RS on the microbiota of younger subjects. The authors of one of these studies, Young et al, speculated that manipulation of the early microbiota may have far greater long -term effects on community composition in light of resilience against long-term diet-induced changes in the microbiota of adults (Young, Roy et al. 2012). In their study conducted in weanling rats, they found that feeding a maize-derived resistant starch could modulate the composition of the colonic microbiota from weaning, and moreover, the increase in proportions of *Bacteroidetes* and *Actinobacteria* from feeding the RS was greater than previously reported in adult studies (Young, Roy et al. 2012). This suggests that modifying the diet at an early age could indeed have a greater effect on microbial community composition. The authors also determined that feeding RS to these young animals resulted in changes in the expression of various host genes, including those involved in the generation of immune suppressing T regulatory cells and the development of colorectal cancer (Young, Roy et al. 2012). Thus, it is likely that such early alteration in microbiota composition imparts physiological responses in the host that could indeed programme long-term health outcomes.

A particular strength of this study was the synchronous collection of both microbial, metabolomic and host transcriptomic data. Such an approach removes some of the ambiguity when trying to interpret the physiological significance of changes in the gut microbial ecology. Limiting the scientific conversation to changes in the relative abundance of "beneficial bacteria" such as lactobacilli and bifidobacteria may no longer be adequate. Future studies could examine the effect of dietary manipulation of the emerging microbiome on potential novel biomarkers which reflect beneficial host outcomes.

2.7.4 Modified starches and infants

The safety of chemically-modified starches, including starch acetate, has been assessed in a series of evaluations conducted by JECFA and the US Select Committee on GRAS Substances (SCOGS). Animal studies provide the major toxicological data upon which these assessments

have been made. In one such study weanling Wistar rats were fed a modified starch at 0, 5, 10, or 25% dietary levels for 90 days. Growth, food intake, efficiency of food utilization and blood biochemistry were similar in test animals to those of controls. In the 25% test group the relative caecum weight was higher than in the controls, and there was a higher number of animals with calcaneous deposits in the renal pelvis. In a two-year rat study using five different chemically modified starches fed at various dietary levels, it was found that a 30% level intake of any of the modified starches led to a slight increase in suburothelial deposits of calcium (de Groot, Til et al. 1974). However, this was not thought to be of any toxicological significance.

Following their analysis of the available data, SCOGS and the FDA concluded that "there is no evidence in the available information on starch acetate that demonstrates or suggests reasonable groups to suspect a hazard to the public when it is used at levels that are now current and in the manner now practiced" (FASEB 1979). The use and safety of modified starches in food for infants and young children has been specifically reviewed (Lanciers, Mehta et al. 1998). While no major concerns have been raised, some authors note a paucity of long term clinical studies and adequate data regarding nutrient absorption and intestinal changes, and suggest that the inclusion of modified food starches in infant products should only be performed prudently and sparingly (Filer 1971; Lanciers, Mehta et al. 1998).

2.8 Prebiotic use during infancy

The concept of prebiotics was first defined in the scientific literature in 1995 by Glen Gibson and Marcel Roberfroid. They defined prebiotics as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" (Gibson and Roberfroid 1995).

Over the last few years, this definition has been frequently revised but three essential core tenets remain – the substance should escape digestion by human digestive enzymes in the upper gastrointestinal tract, be fermented by intestinal microorganisms, and selectively stimulate the growth and activity of those intestinal microorganisms that are associated with beneficial health effects in the host.

With earlier methods of bacterial identification relying on culture and probe based methods, it was generally held that prebiotics predominantly exerted their health benefits by selectively enriching bifidobacterium and lactobacilli numbers. However, as the analytical methods available have expanded, so too has the appreciation that the effects of prebiotics on microbial composition and function are much more complex than previously envisaged. The capacity of prebiotics to confer beneficial health effects on the human host is unlikely to be limited to the selective enrichment of only a select few groups of bacteria. The inability to define an optimal composition for the gut

microbiota has led some to even suggest that the selectivity criterion should be omitted from the definition of prebiotics (Bindels, Delzenne et al. 2015). Other effects of fibre consumption, such as an increase in gut bacterial diversity and the increased production of fermentation end products such as SCFAs, are also likely to have beneficial physiological benefits.

Most commercially available prebiotics are oligosaccharides, a polymer of 3 to 10 simple sugars linked together (Figueroa-Gonzalez, Quijano et al. 2011). The main substrates in this group include the fructans (oligofructose, inulin, fructo-oligosaccharides) and the galacto-oligosaccharides. Fructo-oligosaccharides (FOS) are linear fructose oligomers that contain one glucose unit and 2-4 fructose units bound together by β(2-1) glycosidic linkages (Macfarlane, Macfarlane et al. 2006). One form of FOS can be produced through the enzymatic digestion of sucrose,while inulin-type FOS is derived from the enzymatic digestion of inulin (Bindels, Delzenne et al. 2015). Inulin occurs naturally in many foods, but for commercial purposes, food grade inulin is principally obtained from the chicory plant (Flamm, Glinsmann et al. 2001). Galacto-oligosaccharides are galactose containing oligosaccharides and are mainly produced from lactose utilising the transgalactosylase activity of the bacteria or fungus derived enzyme β–galactosidase (Macfarlane, Macfarlane et al. 2006).

Despite being obtained from relatively low-cost raw materials, the cost of manufacturing many of the above-mentioned prebiotics is high. Many are synthesised through enzymatic processes where the final nature of the formed oligosaccharide is dependent upon the enzyme source, reaction conditions and the nature of the substrate. The low yields associated with such enzymatic production, along with the need for further purification and analyses to ensure end quality, contribute to the high costs (Figueroa-Gonzalez, Quijano et al. 2011).

Eventually, it emerged that other dietary components including forms of resistant starch, could potentially fulfil the criteria required to be considered a prebiotic (Fuentes-Zaragoza, Riquelme-Navarrete et al. 2010). Arguably, it is during infancy that the intake of prebiotics might have the greatest impact on long term composition of the gut microbiota, and also the greatest potential to influence host immune function. However, there is still insufficient clinical data and an absence of formal recommendations from governing authorities regarding the use of prebiotics during infancy, and particularly during the weaning period (Vandenplas, Zakharova et al. 2015). Despite an abundance of literature to support the possible health benefits of early modification of gut microbial composition, there are very few long term clinical studies that have been performed to evaluate the potential effects of prebiotics administered in early childhood on such parameters as later immune and metabolic function and final composition of the gut microbiota (Fanaro, Marten et al. 2009) .

Substrates such as inulin, FOS and GOS, by virtue of their small molecular size, have the potential to exert a significant osmotic effect, resulting in the sequestration of fluid into the colonic lumen.

In vitro studies using faecal slurries, have also demonstrated that FOS and GOS are rapidly fermented by human gut microbiota, particularly in comparison to longer chain carbohydrates which are fermented much more slowly. Rapid fermentation is associated with the accumulation of gas and the rapid distension of the colonic lumen, potentially resulting in bloating, abdominal discomfort and increased flatulence (Shepherd, Lomer et al. 2013).

Keeping these limitations in mind, HAMS could offer several advantages over currently available infant prebiotics. HAMS is not osmotically active, is less rapidly fermented and can likely be produced for less cost on an industrial scale (Nordgaard, Mortensen et al. 1995). Starches, including HAMS and mHAMS have also been shown to be relatively stable when stored in their bulk form (as a dry powder) for indefinite periods of time (Ashwar, Gani et al. 2016). The potential preventative health effects of HAMS on colorectal cancer and its effects on metabolic indices such as plasma lipids, insulin sensitivity and adiposity have been examined in several human and animal studies (Robertson, Bickerton et al. 2005; Keenan, Zhou et al. 2006; Conlon, Kerr et al. 2012; Aryana, Greenway et al. 2015). Fewer clinical trials have investigated the prebiotic potential of HAMS and none have examined the capacity of HAMS to function as a prebiotic during weaning (Ordiz, May et al. 2015).

2.8.1 Clinical effects of prebiotics in infants

By its very definition, the intake of prebiotics is associated with an increase in luminal bacterial mass and an increased production of SCFAs and other fermentation metabolites. This increase in the concentration of small molecules within the colonic lumen can cause a clinically significant osmotic effect, increasing the volume of intestinal contents.

An increase in faecal mass can also stimulate peristalsis through intestinal distension and influence intestinal motility (Niittynen, Kajander et al. 2007). Consistent with these observations is that in several studies where prebiotics have been added to infant formula, an increase in the frequency of defecation and a softer consistency in the stool has been noted (Mugambi, Musekiwa et al. 2012; Orel and Reberšak 2016). The administration of prebiotics thus appears to reduce the prevalence of underlying functional constipation rather than precipitating diarrhoea.

A meta-analysis, published in 2009, reviewed 11 randomised controlled trials involving prebiotic supplementation in full-term neonates and concluded that prebiotic supplemented formula is well tolerated and can increase stool colony counts of bifidobacteria and lactobacilli (Rao, Srinivasjois et al. 2009). It must be noted that the prebiotics administered in the included studies were all forms of oligosaccharides. The meta-analysis also found that there was no evidence that prebiotics had any effect on growth (Rao, Rao et al. 2009). In 2011, a multicentre, placebo controlled, double blind randomised trial involving 1,130 healthy, term infants also found that, in comparison to

standard infant formula, a formula supplemented with a mixture of prebiotic oligosaccharides had no effect on growth outcomes (Piemontese, Gianni et al. 2011).

2.8.1.1 Prebiotics and risk of infection

The potential of prebiotics to protect against infections in infants has also been the subject of investigation. An open label randomized placebo controlled trial by Bruzzese et al, 2009 recruited nearly 300 healthy infants to either receive a GOS/FOS supplemented or a standard infant formula prior to the commencement of solids. In total, 136 infants were allocated into the GOS/FOS group and 146 into the control group. Following 12 months of study involvement, there was a reduced incidence of acute diarrhoea in children in the GOS/FOS group compared to the control group (Bruzzese, Volpicelli et al. 2009). There was also a significant reduction in the number of children with more than three episodes of upper respiratory tract infections per year in the group receiving GOS/FOS (Bruzzese, Volpicelli et al. 2009). In a study by Arslanoglu et al, 259 newborns with a parental history of atopy were randomized to either receive regular formula or a formula supplemented with a combination of GOS and FOS. The participants and the researchers were blinded as to which formula each participant received. In the first six months of life those infants who had received the prebiotics had fewer episodes of all types of infections combined ($p=0.01$) (Arslanoglu, Moro et al. 2007). Follow up continued until two years of life, when it was also demonstrated that infants who were in the prebiotic group had a significantly lower incidence of allergic manifestations and fewer episodes of physician-diagnosed overall and upper respiratory tract infections ($P<0.01$). Given that the prebiotics were only administered during the first six month period, the lasting effects of the intervention suggest that the immune-modulating effects of the prebiotic might have been the principal mechanism of providing protection against the development of infections (Arslanoglu, Moro et al. 2008). However, in 2011, another multicentre study involving the administration of prebiotics to healthy infants within the first year of life found no effect of prebiotic administration on reducing the number of febrile episodes (van Stuijvenberg, Eisses et al. 2011).

In terms of protection against diarrhoea, the fermentation of a prebiotic is associated with a lowering of intestinal pH and a reduction in the growth of pH sensitive pathogenic organisms (de Vrese and Marteau 2007). Rotavirus is one of the leading cause of severe diarrhoea among infants and young children. Recently it has been demonstrated that a daily supplement of a GOS/FOS prebiotic mixture showed beneficial effects on rotavirus-induced gastroenteritis in a neonatal rat model, “modulating clinical parameters and immune system response early in life.”(Rigo-Adrover, Saldaña-Ruíz et al. 2016)

Two clinical studies have specifically examined the efficacy of prebiotics against acute gastroenteritis. In the first of these studies, a two arm randomized, double blinded placebo controlled clinical study, a proprietary polyphenol-based prebiotic (Aliva) was administered to

randomly assigned participants suffering from acute diarrhoea (Noguera, Wotring et al. 2014). The participants included adults and children older than 2 years of age. In total, 200 participants received the prebiotic supplement while 100 were randomized to receive a placebo. Both the prebiotic and the placebo were combined with the oral rehydration solution. The study found that administration of the prebiotic significantly shortened the time to the last unformed stool. It must be noted that this particular prebiotic has not been tested in infants. In another study, conducted in Perth, Australia, a mixture of probiotics and prebiotics (Raftilose) was provided to 493 children aged between one and three years who were attending government childcare centres (Binns, Lee et al. 2007). This study was not designed to determine the therapeutic effect of the prebiotic mixture during an episode of acute infectious diarrhoea, but rather whether it had a preventative effect. The primary endpoint for the study was the number of days a child was reported to have more than four stools in one day over the duration of the study. The study found that on an intention to treat analysis, the children consuming the prebiotic mixture had a 20% reduction in the number of days they experienced more than four stools in one day (Binns, Lee et al. 2007).

2.8.1.2 Prebiotics and risk of allergies

There have been several recent systematic reviews addressing the role of prebiotics for the prevention of allergies in infants. The majority of the studies identified in these reviews examined the use of prebiotics in milk formula; that is, their use in non-exclusively breast-fed infants. Among the professional organisations which publish guidelines regarding paediatric health policies there are no consensus recommendations regarding the use of prebiotics in the prevention of allergies.

The recently published European Academy of Allergy and Clinical Immunology (EAACI) Food Allergy and Anaphylaxis Guidelines state that “there is no evidence to recommend prebiotics or probiotics or to prevent food allergy” (Muraro, Halken et al. 2014). Although both The European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPHGAN) and the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) Committee on Nutrition concede that some benefits may be gained through the administration of prebiotics in infant formula, additional studies are still required before recommendations regarding routine administrations can be made (Szajewska, Guarino et al. 2014). However, recently published guidelines by the World Allergy Organisation (WAO) do recommend prebiotic supplementation in non-exclusively breast-fed infants, who are both at high and at low risk of developing allergies (Cuello-Garcia, Fiocchi et al. 2016). The committee did not extend this recommendation to exclusively breast-fed infants.

More recently, a multicenter randomised double blind trial found that the provision of a formula supplemented with oligosaccharides for the first 26 weeks of life, resulted in both an immune modulatory effect and a reduction in allergic manifestations in infants who had been introduced to solids >18 weeks from birth (Boyle, Brown et al. 2015).

2.8.1.3 Prebiotics in weaning food

Several studies have also examined the role of prebiotics once complementary feeding has commenced. In a 2003 study, 56 healthy infants received either 0.75 g of FOS per serving of cereal or a placebo for a 28 day period (Moore, Chao et al. 2003). The primary outcome was gastrointestinal tolerance as measured by parent recordings of stool patterns, and secondary outcomes included measurements of cereal intake and anthropometric measurements. The findings revealed that FOS-supplemented cereal was well tolerated, and that its consumption led to softer and more regular stools (Moore, Chao et al. 2003).

In a double blind randomized trial involving 20 infants, the effect of supplementing weaning foods with a mixture of GOS and FOS on the proportion of bifidobacteria in the stools was examined. While it was demonstrated that the enrichment of solid foods with a mixture of prebiotics increased stool bifidobacteria proportions, there was no information on growth, overall microbial composition, or markers of immune function (Scholtens, Alles et al. 2006).

Fanaro et al examined the effect of low doses of GOS during weaning on the abundance of members of the faecal microbiota in 159 healthy infants (Fanaro, Marten et al. 2009). In contrast to the study by Scholtens, other parameters were also measured including infant growth, stool consistency, and water balance. The results confirmed that when GOS was consumed for 18 weeks as a follow-on formula together with other weaning foods, it had a bifidogenic effect. The treatment was well tolerated and there was no negative impact on water or electrolyte balance. Concerns that the increased intake of dietary fibre might lead to growth inhibition through the binding of essential nutrients were not supported by the study findings (Fanaro, Marten et al. 2009).

In a study by Saavedra et al, 123 healthy infants attending a large metropolitan day-care centre between the ages of 4 and 24 months were enrolled in a randomized study where they either received a commercially available infant cereal alone or the same cereal supplemented with oligofructose (Saavedra, Tschernia et al. 1999). These healthy children had already been consuming cereal prior to enrolment. Once enrolled, the children remained on the study for as long as the cereal was consumed. Several measurements were taken during the course of the study including height, weight, gastroenterological symptoms, bowel movement frequency and consistency. Antibiotic intake and health profession documented infections were also recorded. The results indicated that cereal consumption was similar in both groups and that there was no difference in growth parameters, stool frequency, or consistency. However, there were highly significant differences in relation to the day-care absenteeism rate due to reported diarrhoea (higher in the control group), and a highly significant decrease in medical attention-seeking during a diarrhoeal episode in the oligofructose-supplemented group (16.13% v control 24.3% $P<0.05$). Duggan et al studied whether the addition of oligofructose with or without zinc to infant cereals

could reduce the prevalence of diarrhoea in high risk Peruvian children between 6 and 12 months of age (Duggan, Penny et al. 2003). The authors found no differences between the supplemented and non-supplemented groups in relation to prevalence of diarrhoea or use of antibiotics (Duggan, Penny et al. 2003). However, the children were at high risk of a disrupted intestinal microbiota and their capacity to ferment the prebiotic may have been affected. The extent of fermentation was not measured in the study.

Firmansyah et al (2011),studied the effects of supplementation of a cereal weaning food with oligofructose and inulin on the immunological response to the measles vaccine. In this double blinded, placebo controlled trial, 50 infants aged 7-9 months were randomized to either receive a cereal supplemented with oligofructose and inulin or a standard cereal. Following a four week period in which the participants received at least one serving of the cereal per day, the children received a live attenuated measles vaccination. The cereal was then continued for a further six weeks. At week 10 antibody levels (anti measles IgG and IgM) were measured. Similar to the Duggan et al study, there were no differences in the mean intake of cereal between the two groups. However, there was a significant difference in anti measles IgG post immunization from baseline measurements, with the supplemented group having a 6.6 fold increase compared to the 4.2 fold increase in the control group ($P<0.03$).

Synbiotics, defined as mixtures of prebiotics and probiotics that influence host health by improving the survival and implantation of specific bacteria in the gastrointestinal tract, offer another method to manipulate the gut microbiota. Milk formulation (400 mL/day) containing a synbiotic mixture of *B.longum, L.rhamnosus*, FOS and GOS and long chain polyunsaturated fatty acids was administered to 12-month old healthy toddlers for a period of 12 months (Firmansyah, Dwipoerwantoro et al. 2011). Between 12 and 16 months following the commencement of the study, weight gain was greater in the synbiotic group compared to the control group ($p<.025$). During this period, there was also a significant increase in faecal abundance of lactobacilli and enterococci. This reflects one of the proposed main benefits of synbiotics, the increased persistence of probiotics in the gastrointestinal tract. More *in vivo* studies comparing the effect of synbiotics with respect to their individual components are needed before it can be claimed that the symbiotic has superior effectiveness to either the probiotic or the prebiotic (Figueroa-Gonzalez, Quijano et al. 2011).

2.8.2 Resistant starch as an infant prebiotic?

If the prebiotic properties of resistant starch are replicated in infants, then supplementation of the infant weaning diet with RS may lead to clinical effects similar to those already described with inulin, FOS, and GOS. However, different substrates might have very different effects on the composition and function of the microbiota. Thus, the interaction between the gut microbiota and

the host may depend on the choice of prebiotic. If *in vitro* fermentation studies confirm the bifidogenic properties of resistant starch using infant faecal inocula then larger *in vivo* studies should be considered. Ideally, a placebo controlled, randomized trial with a large sample size will then determine the clinical efficacy of any RS intervention in this age group. More information regarding study design, and primary and secondary outcome measurements may be gained by reviewing those studies already conducted into the effects of FOS/GOS supplementation during the same period of human development.

Recently, another intriguing property of HAMS, not apparently shared with oligosaccharides, has been described. It has been demonstrated that HAMS may directly interact with intestinal epithelial and dendritic cells to induce a regulatory immune phenotype independent of its effects on the microbiota (Bermudez-Brito, Rosch et al. 2015). This may occur through direct stimulation of immune cells by binding of the starch to pattern recognition receptors TLR-2 and TLR-5. Previous studies have demonstrated that such TLR-2 stimulation by dietary fibres may be involved in the preservation of intestinal barrier function and the induction of T regulatory cell response. Such results emphasise the need to measure immune function during any proposed HAMS intervention study.

2.9 Resistant Starch in the treatment of childhood diarrhoea

Resistant starch, and in particular HAMS, has already been the subject of clinical trials in older children, examining its role in the colonic salvage of water during acute diarrhoea. Acute diarrhoea is defined by the WHO as the passage of loose or watery stools, with or without blood, at least three times in a 24 hour period (WHO 2005). One of the major forms of acute diarrhoea is acute watery diarrhoea, a condition which can cause rapid severe dehydration due to the significant loss of fluid. Indeed, in children less than five years of age, acute diarrhoea is second only to pneumonia as the leading cause of mortality, with an estimated 1.7 billion cases and 0.76 million deaths yearly (Singh, Yang et al. 2014). Although the vast majority of these deaths occur in low income countries, acute infectious diarrhoea remains a major cause of morbidity amongst children living even in high income countries (Freedman, Pasichnyk et al. 2015).

The incidence and mortality from diarrhoea and pneumonia vary by age, as illustrated in Fig 2-4. Younger children are particularly vulnerable to adverse outcomes from acute infectious diarrhoea, with 72% of the estimated number of deaths associated with diarrhoea occurring in the first 2 years of life (Walker, Rudan et al. 2013) Notably, the incidence of diarrhoea peaks at age 6-11 months and then decreases with age. This peak appears to coincide with the introduction of solids into the child's diet.

Fig 2-4 has been removed due to copyright restrictions

Figure 2-4 Distribution of cases of and deaths from diarrhoea and pneumonia in children aged <5 years of age (Walker, Rudan et al. 2013).

There has been a significant reduction in the numbers of children dying from diarrhoea globally. Over twenty five years ago the childhood mortality from acute diarrhoea was approximately 12 million per year (Singh, Yang et al. 2014). In 2011, it was estimated that globally, approximately 700,000 children died from acute diarrhoeal events (Walker, Rudan et al. 2013). However, these numbers remain unacceptably high. In 1979, the oral rehydration solution (ORS) was first recommended by the WHO for use during mild to moderate episodes of acute diarrhoea. Its introduction is one of the major contributing factors to the subsequent dramatic decrease in global diarrhoea related deaths. The key properties of the ORS include its capacity to be administered by the primary caregiver, in the home under the guidance of a health care professional and its relative accessibility in terms of cost and availability.

However, despite such an impressive reduction in mortality, the uptake of ORS has been disappointing and this may partly account for the continued number of potentially preventable deaths. A major reason for this poor uptake is that the traditional ORS fails to reach endpoints of significance to the caregiver, such as a reduction in stool output or a reduction in the duration of the diarrhoeal illness (Bhattacharya 2000). Dehydration remains one of the major causes of death during acute infectious diarrhoea and the fact that the ORS effectively treats this, but is still not being used at appropriate rates, emphasises a residual need for novel treatments that will satisfy caregiver perception, of what qualifies as a beneficial intervention. Combining resistant starch with the ORS has been proposed as one such intervention (Ramakrishna, Venkataraman et al. 2000). However, to understand how an ORS-RS intervention could contribute to the treatment of diarrhoea it is necessary to review both the pathophysiology of acute diarrhoea, and intestinal electrolyte and fluid transport in the normal intestine.

2.9.1 Pathophysiology of acute diarrhoea

Diarrhoea essentially results when the absorptive and secretory function of the gut is out of balance either through inadequate absorptive function or through excessive secretion (Singh, Yang et al. 2014). The daily loss of only around 150 mL of fluid in stools, despite 8-9 litres of fluid entering the intestinal lumen is a testament to the enormous absorptive capacity of the intestinal epithelium (Keely, Montrose et al. 2009). In a state of good health, the small intestine absorbs the majority of this daily luminal fluid. In a healthy gut, absorption and secretion of fluid by enterocytes is governed by several intestinal ion transport mechanisms, namely chloride secretion, electroneutral NaCl absorption, and electrogenic Na absorption (Keely, Montrose et al. 2009).

There is regional division of function, with epithelial cells in the villus of the small intestine and upper crypt of the colon primarily involved in absorption, while those in the lower crypt are primarily secretory in function (Singh, Yang et al. 2014). The epithelial cell itself can be divided into two regions, the apical (brush-border) and basolateral (serosal) membranes. Intestinal chloride secretion across the intestinal epithelium is perhaps the most important mechanism for fluid secretion throughout the gastrointestinal tract (Field 2003). Absorption of sodium from the lumen and its exit from the basolateral side of enterocyte is critical for absorption of water which passively follows the osmotic gradient created by the cation (Field 2003). The Na^+K^+ ATPAse pump on the basolateral membrane of the enterocyte provides the driving force for net transcellular movement of Na^+, K^+, and HCO3 along the length of the small intestine. Sodium and chloride absorption occur through the coordinated regulation of apical membrane sodium hydrogen exchangers (NHEs) and chloride, bicarbonate exchangers (Kunzelmann and Mall 2002). These are, in turn regulated by various second messenger pathways and intra-cellular signal transduction. Fig 2-5 illustrates the major components of the intestinal sodium absorptive cell.

Fig 2-5 has been removed due to copyright restrictions

Figure 2-5 Intestinal Sodium Absorptive cell (Singh, Yang et al. 2014)

Many bacterial and viral pathogens which cause diarrhoea in children can activate intracellular messengers, such as cAMP, which stimulate chloride secretion from crypt cells and inhibit the neutral coupled sodium chloride absorption (Field, Fromm et al. 1972). For instance, in cholera, the cholera toxin increases the production of cAMP which, in turn, leads to an increase in the active secretion of chloride into the lumen and an inhibition of NaCl absorption through the inhibition of the NHE dependent NaH pump (Subramanya, Rajendran et al. 2007). The cumulative effect of these changes is a significant loss of fluid into the lumen resulting in up to seven litres of diarrhoea per day (Binder 2010). In resource-poor countries, where intravenous fluids are not accessible, it is not surprising that cholera can kill rapidly.

2.9.1.1 Physiological basis of the oral rehydration solution

During the 1950s, it was discovered that through the actions of a particular co-transporter, sodium could also be transported into the cell by coupling its movement with the transport of various nutrients, mainly sugars and amino acids (Crane 1960). This transporter was later defined as SLGT1. A key observation was made that this Na^+ dependent glucose or amino acid transport was not inhibited by an increase in second messengers such as cAMP (Field, Fromm et al. 1972). This became the physiological basis for the oral rehydration solution (Binder, Brown et al. 2014).

The first large clinical trial of an oral rehydration solution was performed in patients with active cholera during the Bangladesh War of Independence (Mahalanabis, Choudhuri et al. 1973). It was found that the addition of glucose to orally administered salt solutions could indeed promote the absorption of water in patients with cholera and result in a significant reduction in mortality. A major drawback of the traditional ORS is that it does not shorten the duration of the diarrhoea or reduce stool volume, and this may have limited its uptake by caregivers, due to perceptions that it was ineffective (Binder, Brown et al. 2014).

SLGT1, the small intestinal apical membrane transporter protein that is involved in the absorption of glucose, and which is critical to the efficacy of the ORS, is not present within the large intestine (Binder 2010; Binder, Brown et al. 2014). However, SCFAs, by-products of the microbial fermentation of predominantly dietary substrates which may have otherwise remained unabsorbed in the intestine, are avidly absorbed by colonic epithelial cells (Binder, Brown et al. 2014). In the 1980s, researchers determined that the absorption of SCFAs could also facilitate the movement of sodium into the colonic enterocyte (Binder and Mehta 1989).

2.9.1.2 Coupling of SCFA and Na across the apical membrane

In a series of elegant *in vitro* transport studies using the distal rat colon, it was found that active Na^+ and Cl^- absorption was enhanced by SCFAs, and this was achieved through the coupling of Na^+ H^+ exchange with both butyrate-HCO3 exchange and butyrate Cl exchange (Krishnan, Ramakrishna et al. 1999; Binder 2010). This model of SCFA coupled Na^+ absorption is illustrated in Fig 2-6.

Importantly, researchers discovered that SCFA stimulated Na^+ absorption was not inhibited by cAMP, highlighting an absorptive pathway that could be utilised during the therapy of diarrhoeal diseases such as cholera (Binder, Brown et al. 2014). It was later determined that the differential effects of cAMP on HCO3 stimulated Na absorption versus butyrate stimulated Na^+ absorption was due to different isoforms of sodium hydrogen exchangers (NHEs) (Binder, Brown et al. 2014). While cAMP is known to inhibit the NHE3 isoform, it was shown to stimulate the NHE2 isoform, the exchanger responsible for butyrate stimulated Na^+ absorption (Krishnan, Rajendran et al. 2003).

Fig 2-6 has been removed due to copyright restrictions

Figure 2-6 Model of SCFA stimulation of Na and Cl absorption in colonic epithelial cells (Binder 2010).

2.9.1.3 Utilising SCFA to enhance the absorption of water from the colon

The colon has a significant latent capacity for water reabsorption. The healthy adult colon can reabsorb 1.5 litres of fluid per day, but it is known that under certain circumstances it can even triple this capacity to approximately 5 litres (Binder 2010; Binder, Brown et al. 2014). Thus SCFA enhanced colonic Na^+ absorption could be a potent mechanism of reabsorbing fluid during episodes of acute diarrhoea. Ramakrishna and colleagues recognised that the dietary provision of high amylose maize starch (HAMS) could provide the mechanism to increase luminal SCFAs (Ramakrishna, Subramanian et al. 2008). It was also thought that while the majority of the HAMS would reach the colon undigested, a small component may be digested to glucose in the small intestine, thus also facilitating small intestinal sodium absorption, replicating the mechanism of action of the conventional ORS (Binder 2010). The researchers speculated that for the treatment of acute infectious diarrhoea, this resistant starch intervention could be combined with the traditional ORS to achieve more favourable endpoints such as a reduction in the duration of diarrhoea and a reduction in stool volume (Binder, Brown et al. 2014).

A series of experiments were designed to test this principle. The initial experiments demonstrated that *in vitro* incubation of RS with stool from patients with acute cholera could indeed result in the production of SCFAs. Following this, a randomised controlled trial was performed in adults suffering from acute cholera, in which three different ORS were compared: standard ORS, rice flour based ORS, and high amylose maize RS (Ramakrishna, Venkataraman et al. 2000). It was found that RS-ORS was superior to the other two ORS formulations with regard to time to formed stool. Fig 2-7 illustrates normal fluid balance within the large intestine and the changes that might occur with cholera infection and subsequent treatment with the ORS or a combined resistant starch-ORS intervention.

Fig 2-7 has been removed due to copyright restrictions

Figure 2-7 Fluid balance within (a) normal subjects, (b) patients infected with cholera, (c) patients receiving the ORS and (d) patients receiving the combined resistant starch-ORS (Binder 2010).

As children are particularly vulnerable to the adverse effects of acute infectious diarrhoea, a randomised controlled study was conducted to determine the efficacy of a combined RS-ORS (HAMS as the RS intervention) in comparison to the traditional ORS for the treatment of acute non-cholera diarrhoea in children from six months to three years (Raghupathy, Ramakrishna et al. 2006). The study confirmed that compared to the isotonic WHO-ORS formulation that was recommended at the time of publication, the combined RS-ORS intervention significantly shortened the duration of the diarrhoea in comparison to the standard ORS preparation.

In the 1990s, it emerged that a hypo-osmolar formulation of the ORS was associated with a lower incidence of hyponatraemia and greater water absorption in comparison to standard ORS for the treatment of acute non-cholera associated diarrhoea (Hunt, Elliott et al. 1992; Mahalanabis, Faruque et al. 1995). A meta-analysis found that among children with acute diarrhoea, the reduced osmolarity ORS decreased stool volume and the likelihood of unscheduled intravenous fluid therapy in comparison to the standard solution (Hahn, Kim et al. 2001).

Consequently, in 2004 the WHO changed its ORS formulation to incorporate these new findings (Hahn, Kim et al. 2001). Ramakrishna et al performed a further randomized clinical study comparing the efficacy of the RS-ORS preparation to the superior hypo-osmolar version of the ORS in adult patients with acute cholera (Ramakrishna, Subramanian et al. 2008). The authors found that even when compared to the hypo-osmolar ORS, the combined RS-ORS preparation led to superior clinical outcomes, with a 55% reduction in diarrhoea duration. Other fermentable fibres have also been investigated in relation to their potential to improve the treatment of diarrhoea. Green banana is a natural source of RS2. Rabbani et al found that the administration of green banana reduced the amount of stool, diarrhoea duration, and oral rehydration requirements in boys aged 5-12 months with persistent diarrhoea (Rabbani, Teka et al. 2001). Alam et al (2015), also investigated the effects of combining the ORS with partially hydrolysed guar gum (PHGG) in severely malnourished children with watery diarrhoea. Like HAMS, PHGG is a fibre which is fermented by gut microbes to produce SCFA, which can, in turn, promote the absorption of water from the colon. The study, conducted in children aged from 6 to 36 months, found that PHGG added to hypo-osmolar ORS reduced duration of diarrhoea and stool output (Alam, Ashraf et al. 2015). No head-to-head studies have been conducted to determine the preferred fermentable fibre that could be added to the ORS in order to improve its efficacy.

It is not known from what age children could receive and benefit from any resistant starch – ORS intervention. As yet, clinical trials involving the administration of HAMS to children with acute diarrhoea have not been performed in infants. A major strength of the current WHO ORS formulation is that it is available for use even in early infancy. An increase in diarrhoea incidence occurs during weaning when infants are no longer exclusively breast-fed. Contamination of complementary foods or the intake of diets that may contain inadequate nutritional value contribute towards this risk. If a HAMS-ORS intervention is to replace the current ORS, then it would need to demonstrate efficacy and safety within this age group.

Age may certainly be a factor in determining the efficacy of administering a malabsorbed carbohydrate during an episode of infectious diarrhoea. Using three week old and three month old pigs that had been infected with an infectious pathogen, researchers demonstrated that the older pigs had less diarrhoea than their three day old counterparts when given a malabsorbed carbohydrate (Argenzio, Moon et al. 1984). The authors speculated that in the older pigs, the

carbohydrate reaching the colon was completely fermented, producing increased amounts of colonic SCFAs, while the carbohydrate passed unchanged through the colon of the younger pigs, making no contribution to fluid homeostasis. The authors concluded that the development of the capacity to ferment a substrate and absorb the resulting SCFAs was critical in mediating the colon's capacity to compensate for fluid loss during an acute episode of infectious diarrhoea (Argenzio, Moon et al. 1984).

2.10 Zinc and resistant starch

Zinc deficiency is another factor contributing to the burden of diarrhoea in low income countries. A number of systematic reviews have demonstrated that in children aged over 6 months, zinc supplementation at 20mg per day for 10 days at the time of an acute diarrhoeal episode may reduce the incidence of further episodes of diarrhoea (Lazzerini 2016). In many low income countries a child may suffer from more than three episodes of acute diarrhoea in one year, leading to a vicious cycle of micronutrient deficiency and further diarrhoeal episodes.

There is evidence to suggest that fermentable fibres such as resistant starch may promote the absorption of divalent cations from the large intestine (Younes, Demigne et al. 1996; Lopez, Coudray et al. 1998). The mechanism is multifactorial, with a reduction in intestinal pH and a resultant increase in mineral solubility likely contributory factors, If in addition to its potential beneficial effects on the microbiome and colonic water salvage it was demonstrated that resistant starch increased the absorption of zinc from the infant colon, then an RS based intervention could gain even more traction with international health agencies, and potentially achieve a prominent role in global child health policy. The potential for zinc to be absorbed by the human colon has not been reviewed previously and will be examined in further detail in Chapter 5 of this thesis.

Since embarking on this thesis, a study investigating whether a resistant starch intervention (HAMS) could increase the absorption of zinc in stunted children living in Malawi has been published. The results of the study do not support the facilitation of zinc absorption through an RS intervention. Nevertheless, this thesis will consider whether preceding zinc deficiency, which was absent in the Malawi participants, could yet influence the capacity of RS to affect the absorption of zinc. As it would be cost-prohibitive and ethically challenging to perform such a study on humans, a weanling rodent model will be used to examine this question. I will also consider whether preceding zinc deficiency will influence the capacity of the rodent microbiota to ferment the dietary RS.

2.11 Aims of thesis

Thus, the objectives of my doctoral research are to:

- **Determine if the faecal inocula of young infants (pre-weaning and early-mid weaning) possess the capacity to ferment a high amylose maize starch and a modified high amylose maize starch.**
- **Characterise the changes in the microbial profile that are associated with any such fermentation.**
- **Determine if there is any difference in the parameters of fermentation (production of SCFA and changes to microbiota) following incubation of weaning infant inocula with HAMS in comparison to modified HAMS**
- **Review the evidence for the colonic absorption of zinc**
- **Using, a weaning rodent model, determine whether the capacity of HAMS to promote zinc absorption by altering colonic fermentation is affected by a preceding zinc deficiency.**
- **Using a weaning rodent model, determine the effect of a preceding zinc deficiency on the fermentation of HAMS.**

Chapter 3 Fermentation of Resistant Starch by faecal inocula of infants

3.1 Abstract

In adults, the fermentation of high amylose maize starch (HAMS), a resistant starch, by resident gut microbiota confers a range of health benefits to the human host. Little is known about the degree to which the microbiota of the young infant can ferment HAMS and a commercially available modified HAMS (mHAMS). Thus the aim of this study is to determine if the faecal inocula of young infants (pre-weaning and early-mid weaning) possess the capacity to ferment HAMS and mHAMS

Methods: Faecal samples were collected from 16 infants (aged 3-4 months) pre and post the commencement of solids (weaning). Using *in vitro* batch fermentation methodology, the fermentation of HAMS and mHAMS by infant faecal inocula was assessed. Only weaning infants were assessed for their capacity to ferment mHAMS. To mimic the *in vivo* presentation of the dietary starches to the gut microbiota, the starches underwent an *in vitro* predigestion process prior to incubation with the faecal inoculum. At 24 hours of incubation, values for pH and SCFA production were compared to parallel incubations with a no added substrate, negative control.

Results: Pre-weaning infants had the capacity to ferment HAMS, as evidenced by a significant reduction in pH ($p<0.05$) and a significant increase in the production of total SCFAs ($p<0.05$) at 24 hours when compared to parallel negative control incubations. However, the fermentation of HAMS significantly increased after the commencement of solids. In the weaning infants, it was evident that they also had the capacity to ferment mHAMS. There was no significant difference between the fermentation of mHAMS and HAMS by the weaning infant faecal microbiota.

Conclusions: HAMS can be fermented by pre-weaning infant faecal inocula to some extent; however, this capacity is markedly increased after the commencement of solids. Weaning infants also have the capacity to ferment mHAMS. These findings are promising when considering a role for these starches in any early nutrition intervention. Future *in vivo* studies are needed to determine the effects of consumption of these resistant starches on gut microbial composition and the long term health of the infant host.

3.2 Introduction

It is widely recognised that the prevalence of non-communicable diseases (NCDs) in the Western world has dramatically increased over the last few decades. With the seemingly disparate fields of immunology, microbiology, and nutrition now converging, there is accumulating evidence that perturbed intestinal microbial colonisation during a critical developmental period, partially mediated through changes in modern dietary practices, is interfering with the development of the human immune system and contributing to this rise in NCDs (Andrew, Philip et al. 2011). Short chain fatty acids (SCFA), the by-products of the anaerobic fermentation of predominantly dietary fibre by gut microbes, are critical mediators in host-microbe mutualism and have a direct effect on the development of innate and adaptive immune cell function (El Aidy, Dinan et al. 2015). The term dietary fibre encompasses a diverse range of principally indigestible carbohydrates of plant origin that are resistant to digestion and absorption in the small intestine and are thus available for fermentation by gut microbes (Slavin 2013).

A dramatic reduction in fibre intake in the Western diet may be adversely affecting the composition and metabolomic function of the resident gut microbiota, thus interfering with appropriate development of the human immune system (Mathers and Wolever 2009; West, Renz et al. 2015). In light of the resilience against long term dietary manipulation of the adult microbiome and the relative plasticity of the infant's emerging immune system, it might be expected that if dietary fibre interventions were performed earlier in the human lifespan, there might be a far greater beneficial health impact (Voreades, Anne et al. 2014). In addition to the basic conundrum of a 'normal healthy gut microbiota' not being clearly defined, the duration of the developmental window within which manipulation of the host microbe interaction can lead to sustained changes in immune function is not known. It could also be expected that the inadequate provision of appropriate microbial stimuli during this as yet undefined period may lead to pro-inflammatory response patterns, which once established may not be amenable to change (West, Renz et al. 2015).

The consumption of resistant starch (RS)- one form of dietary fibre, can result in increased production of SCFAs in the colon and possibly the rectum of humans (Bird, Brown et al. 2000) as evidenced by analyses of faecal content (Bird, Brown et al. 2000). Most studies which have demonstrated this have been performed in adults (Zaman and Sarbini 2015). RS is defined as the sum of starch and products of starch degradation which have not been absorbed in the small intestine of healthy individuals which become available for microbial fermentation in the colon in a similar fashion to non-starch polysaccharides (Englyst, Kingman et al. 1992). The increased intake of certain RS can also enhance the growth of particular bacteria that are associated with favourable effects on human health (Fuentes-Zaragoza, Sanchez-Zapata et al. 2011).

However, the potential of a dietary RS intervention in infants has not been adequately explored, due in part to concerns that the relatively immature gut microbial ecosystem of the infant would not have acquired the necessary diversity of bacteria to ferment a complex carbohydrate such as RS (Parrett, Edwards et al. 1997; Palmer, Bik et al. 2007).

In total, there are five classes of resistant starches defined by their physicochemical properties (Englyst, Kingman et al. 1992). Type 1 consists of physically inaccessible starch such as partly milled grains and seeds. Type 2 resistant starch describes native starch granules that have reduced digestibility due to the conformation or structure of the starch granule. High amylose maize starch (HAMS) is a type 2 RS. Other forms of RS2 include green banana flour and raw potato starch, The amylose to amylopectin ratio of starch is a major determinant of its digestibility (Vonk, Hagedoorn et al. 2000; Sajilata, Singhal et al. 2006). Most food starches are predominantly composed of amylopectin (70%),-a highly branched molecule. Amylose, on the other hand, is a relatively small, essentially linear glucose polymer. In high amylose maize starches the amylose:amylopectin ratio is reversed, generating a starch molecule which can be tightly packed together, resisting access to digestive enzymes. A further property of HAMS, is that it retains its resistive properties even during the processing and preparation of many foods (Fuentes-Zaragoza, Riquelme-Navarrete et al. 2010). Obtained through selective breeding of strains of maize that are naturally high in amylose, HAMS has been used in many of the animal and human clinical studies which have demonstrated the varied health benefits of RS (Govers, Gannon et al. 1999; Gower, Bergman et al. 2016).Type 3 RS is retrograded starch obtained by cooking and then cooling the starch. Type 4 resistant starches (RS4) have been chemically modified to reduce digestibility and alter their physicochemical properties. Type 5 resistant starches utilise an amylose-lipid complex to reduce granule swelling during cooking (Hasjim, Ai et al. 2013).

The chemical structure of starch, including the amylose/amylopectin ratio, can not only influence its resistance to digestion in the upper intestine, it can also affect its susceptibility to fermentation by particular microbes (Martinez, Kim et al. 2010). It has been determined that several species of bifidobacteria are adept at starch utilisation, particularly high amylose maize starch (Wang, Conway et al. 1999b). Given that the relative abundance of bifidobacteria is high in infants, more so than in the microbiota of adults, we propose that the faecal inocula of young infants may indeed have the capacity to ferment a high amylose maize starch. If this were the case, a high amylose maize starch intervention could be of value during the initiation of complementary feeding-both functioning as a prebiotic, selectively stimulating the growth of beneficial bacteria such as bifidobacteria, and increasing the generation of SCFAs in the colon.

Modified starches, RS type 4, are often added to foods to impart favourable functional properties which are appealing to the consumer (Miyazaki, Van Hung et al. 2006). Historically, there has been some unease regarding the use of such chemically modified starches in infant food products, due in part to uncertainties regarding the toxicological effect of chemicals used to modify the starch (Lanciers, Mehta et al. 1998). Although advances in food technology have allayed some of these concerns, the chemical modification of starch is still known to increase resistance to digestion in the upper gut, thus increasing the passage of the modified starch into the colon (Wolf, Bauer et al. 1999). In animals and humans, esterification of a high amylose maize starch with a SCFA moiety, can further increase the delivery of the attached SCFA to the colon (Annison, Illman et al. 2003; Clarke, Bird et al. 2007). The microbial enzymes capable of cleaving the ester bond differ from those required to breakdown the native starch backbone (Kaoutari, Armougom et al. 2013). Although international food safety agencies have deemed that most modified starches are safe for use in baby foods, it has been recommended that they should not exceed 5% of the total food component. It is likely that relative to an adult, more dietary modified starch will reach the large intestine of an infant due to a young child's immature pancreatic function (Christian, Edwards et al. 2003). There are no published studies regarding the capacity of the infant's resident gut microbiota to ferment a chemically modified starch, including those that are commercially available.

Even in adults, the *in vivo* assessment of the capacity to ferment starch in the human colon is problematic, due in part to the inaccessibility of stool in the proximal colon, where the majority of fermentation occurs. Direct intubation of the colon has been performed to assist in determining in vivo fermentation capacity for dietary substrates. However, such methods are highly invasive, difficult to carry out, expensive and particularly in the case of young children, might not pass current ethical considerations. Several *in vivo* studies have also utilised breath H_2 or $13CO_2$ measurements as a measure of the digestion and anaerobic fermentation of starch (Olesen, Rumessen et al. 1994; Symonds, Kritas et al. 2004). However, the potential for interference from other fermentable substrates in the diet and the duration of the test are problematic, as is the inability to measure other markers of fermentation such as SCFAs and changes in microbial composition. (Symonds, Kritas et al. 2004). The actual feeding of dietary substrates in clinical trials also presents practical difficulties in controlling for dose and duration of the intervention as well as factoring in the background dietary composition. Furthermore given the increased recognition of the profound effects that early manipulation of the microbiome may have on later life, it may be unethical to participate in the microbial programming of a young child's emerging microbiota without accumulating preliminary *in vitro* data.

In vitro static batch fermentation is a rapid, simple, inexpensive method to assess the fermentability of different substrates which has been widely used for the evaluation of fermentation capacity by both adult and infant faecal inocula (Parrett and Edwards 1997; Christian, Edwards et al. 2003).

The fermentation of a substrate during *in vitro* batch fermentation studies is evidenced by the increased production of SCFAs, a decrease in pH, or differences in microbial composition in those ferments containing the substrate when compared to a negative control. Whilst some studies also measure disappearance of the substrate, this additional step to confirm substrate utilisation is not consistently performed(Parrett, Edwards et al. 1997; Scheiwiller, Arrigoni et al. 2006; Vigsnaes, Holck et al. 2011).

In this study we will use an *in vitro* batch fermentation methodology to determine the capacity for infant faecal inocula to ferment HAMS and a commercially available mHAMS. Negative control samples with no substrate will serve as indicators of the endogenous fermentation capacity of the inocula. It will be necessary to predigest the dietary sources of the RS prior to use in the fermentation studies, as they contain a digestible component of starch and free sugars which in the *in vivo* setting, would not be available to the colonic microbes for fermentation.

Thus, the objective of this study is to evaluate the fermentation of predigested HAMS (high amylose maize starch) and predigested mHAMS (acetylated high amylose maize starch) starch by pre-weaning and early to mid-weaning (weaning) healthy infant faecal inocula using *in vitro* culture methodology. The establishment of fermentation will be determined by detecting a significant increase in total SCFA concentration and/or a reduction in pH relative to parallel negative control incubations.

Specific Aim1: To use in vitro fermentation methodology to determine the capacity of the faecal microbiota of young infants (weaning and pre weaning) to ferment HAMS or mHAMS.

Hypothesis: mHAMS and HAMS can be fermented by infants prior to late weaning.

Specific Aim 2: To determine if fermentation of HAMS is enhanced post introduction of solids.

Hypothesis: the introduction of solid food will enhance the capacity of faecal microbiota to ferment HAMS.

3.3 Materials and Methods

3.3.1 Study design and sample collection

This study has complied with National Health and Medical Research Council (Australia) guidelines relating to ethical conduct in human research and was reviewed and approved by the Southern Health Human Ethics Committee on May 23rd 2013. Written informed consent was obtained from the legally authorized representative of the infants prior to sample collection.

The care-givers of pre-weaning infants were recruited by several methods, including direct

approach at time of delivery or through advertisements placed in both the hospital and in a local child magazine. Both exclusively breast fed infants and mixed fed (breast plus formula fed)infants were recruited. None of the infants or their mothers received antibiotics post-delivery, including between provision of the first and second faecal sample. Other inclusion and exclusion criteria included that the infant was full term at birth with a gestational age of more than 38 weeks; at, or above, the 10th percentile for weight at birth; with no known cardiac, respiratory or gastrointestinal disease and no history of diabetes. None of the infants or their breast-feeding mothers received probiotic supplementation prior to stool collection. There was no requirement for breast feeding mothers to avoid prebiotic use during the study. The care-giver's decision as to when to introduce solids was not influenced by participation in this study. It was also not possible to collect detailed records of food intake by the mothers and infants during this study.

The caregivers were advised that one faecal sample would be required from the infant prior to commencing solids from at least 8 weeks of age, and a second faecal sample would be collected within 6-12 weeks of solids being commenced. Caregivers were advised that the preweaning infant inocula was to be collected prior to the intake of any non milk material, ie porridge or rice cereal. Early to mid-weaning (weaning) was defined as within 12 weeks after giving the first non-milk food.

3.3.2 Outcomes analysis

I will use a paired t-test (with a 0.050 two-sided significance level) to compare negative controls to fermentations containing the test substrate in pre and weaning samples within an individual. Based on the standard error reported in prior literature examining fermentation of other complex polysaccharides in infants (Parrett, Edwards et al. 1997), I determined, using nQuery that a sample size of 10 would have 80% power to detect a difference in means of 6.4 mM (e.g. a First condition mean, of 33.0 mM and a Second condition mean, of 26.6 mM), assuming a standard deviation of differences of 6.0, using a paired t-test. This is based on a standard deviation of 6.8 and a mean of 16.5 (pre-weaning) and a standard deviation of 9.37 and a mean of 32.89 (weaning). Therefore, from my calculation I estimated that I required 10 breastfed infants and 10 formula-fed infants.

3.3.3 Sample collection and processing

A portable freezer was delivered to each participant's house prior to the collection day. Within 15 minutes of an infant passing a motion, stool was collected from a disposable nappy using a sterile container. This container was then placed into an airtight bag and put into the freezer which was set at -20^{0}C degrees. Within two hours, the freezer containing the sample was transported to the laboratory where the samples were immediately processed. Working within an anaerobic cabinet containing 5% H2, 5% CO2 and 90% N2, the faecal samples were homogenized in 50% sterile glycerol (1:1 dilution) and then stored at -80^{0}C until further analysis. The use of frozen samples

compared to fresh samples in fermentation experiments has been previously validated (Rose, Keshavarzian et al. 2009).

3.3.4 Carbohydrates and chemicals

The two resistant starches, HAMS (Hylon VII) and modified HAMS (mHAMS-Crispfilm) were obtained from Ingredion, USA.The modified HAMS had been subject to an acetylation process. Hylon VII is composed of 70% amylose and 30% amylopectin. By measuring the concentration of serum glucose and $13CO_2$ excretion in the breath of adult volunteers six hours post ingestion of Hylon VII (naturally enriched in 13C), Vonk et al estimated that 50% of Hylon VII is digested in the small intestine of healthy adults (Vonk, Hagedoorn et al. 2000). This underlies the need for pre-digestion of the starch to mimic the form in which it will reach the large bowel.

There is no available data indicating what proportion of Hylon VII would be digested in the small intestine of the infant. Crispfilm (CF) has a Hylon VII backbone, but has undergone a further esterification process to form a starch acetate. The degree of acetylation of CF is less than 2.5%, the limit imposed by US FDA regulation. Unpublished correspondence reporting prior external analysis of Crispfilm states an acetyl value of 1.6-1.9%. The faecal inocula of pre and weaning infants can readily ferment lactulose, an insoluble disaccharide, so this substrate was selected as the positive control, i.e. to confirm that viable bacteria were present in the faecal inocula of each subject. Lactulose is an indigestible disaccharide and was not subject to a predigestion process. Hylon VII will be referred to as HAMS and the Crispfilm – the acetylated form of Hylon VII, will be referred to as modified HAMS, mHAMS for the remainder of this manuscript.

3.3.5 Pre-digestion method

As the starches have digestible components, an *in vitro* predigestion step was performed to simulate the digestive action of the infant small intestine prior to the fermentation experiments. There exists significant heterogeneity in the methods used to imitate digestion within the human upper gastrointestinal system, with no consensus regarding the preparation of the initial sample or the use and concentration of different enzyme mixtures to simulate oral, gastric and small intestinal phases of digestion (Woolnough, Monro et al. 2008). The actual conditions of the incubation such as pH and duration may vary considerably between the different methods. Given the relative immaturity of pancreatic function in infants, it is expected that the digestion of starches in the upper intestine of young infants will vary considerably from that of adults (Lebenthal and Lee 1980). However there is almost no prior literature to offer guidance regarding a pre-digestion method which would most closely replicate *in vivo* findings within young children.

I based my *in vitro* digestive method upon one developed by the CSIRO Food Futures National

Research Flagship to mimic starch digestion processes in the adult human upper gastrointestinal tract. The accuracy of this method had previously been assessed by comparing the *in vitro* digestion of a wide variety of foods to results obtained *in vivo* in ileostomy effluent from adult participants. The *in vitro* values strongly correlated with the *in vivo* results, demonstrating the validity of the method (Bird, Usher et al. 2012). In this study, as I was interested in mimicking the small intestinal digestion of starch in infants, I modified the original method which mimicked adult digestive function by adding 10% of the recommended concentration of α-amylase during *in vitro* digestion. This is due to the relative immaturity of pancreatic function in young infants (Butterworth, Warren et al. 2011). No similar adjustment was made to the concentration of amyloglucosidase as its activity is comparable to that of adults from birth (Lee, Werlin et al. 2004). *In vivo*, the oral phase initiates the digestion process. Not only does this involve the mechanical breakdown of food, but it also initiates the hydrolysis of dietary starch through the action of salivary alpha-amylase.

Although salivary alpha-amylase is active in pre-weaning and weaning infants, as their primary oral intake would be in the form of liquid or mashed/pureed food, with no need for chewing, I did not deem it necessary to imitate exposure to salivary alpha-amylase prior to the simulated gastric phase of digestion.

Briefly, 5 g of the test starch was accurately weighed into tubes (8) and incubated with 25 mL of pepsin (1 g/mL, made up in .02 mol HCl/L; pH 2) for 30 minutes at 37 degrees. This was to replicate the gastric phase of digestion, where digestion of protein by pepsin occurs in a highly acidic environment. *In vivo*, as the food bolus leaves the stomach and enters the proximal small intestine, it encounters a more alkaline environment due in part to pancreatic secretions. During *in vitro* digestion, this phase of digestion was replicated by neutralisation with sodium hydroxide (.02M) and incubation with 25 mL of an amyloglucosidase (200 U/mL) and alpha-amylase (1.5 U/mL) solution for 5 hours in a shaking water bath at 37^0C. Following the incubation, contents were pooled and transferred into a beaker containing 80% ethanol and left overnight. The supernatant was then removed and the residue was washed with 80% ethanol. This was then centrifuged (2000 x g for 10 minutes), followed by the removal of the supernatant. Washing with alcohol, centrifuging, and removal of the supernatant was repeated four times. This washing with aqueous alcohol was to remove free sugars in the digested sample. Following air-drying in the fume hood for another 24 hours, the residue was collected for use in the small-scale batch *in vitro* fermentation method–described below. Only one batch of pre-digestion was performed for each substrate and this provided sufficient amounts for all the required incubations. This eliminated the chance of 'between batch' variation. Based on calculations, performed by CSIRO, predigestion of the HAMS using the infant digestion methodology, resulted in an 80% resistant starch component. This compares to a 50% resistant starch component with the adult predigestion method.

3.3.6 Small-scale in vitro fermentation

For each *pre-weaning* infant sample, there were three groups of incubations: HAMS, Lactulose and the negative control (blank). The fermentation experiments were performed in triplicate for each donor/substrate mixture. There was insufficient faecal material available to examine the fermentation of mHAMS by pre-weaning infant faecal inocula. However, for *weaning* samples, due to the increased amount of stool available from a single collection, the fermentation of mHAMS (i.e. Crispfilm), was able to be examined. Where faecal inocula volume was insufficient to inoculate all incubation tubes, the lowest priority was given to inoculating the 0 hour lactulose tubes. The *in vitro* fermentation method was based on the technique described by Edwards et al (Edwards, Gibson et al. 1996) and Goni et al (Goñi and Martin-Carrón 1998). Briefly, 100mg of pre-digested starch residue was weighed into triplicate 15 mL sterile culture tubes together with 8-10 sterile 2.5 mm glass beads and 9mL of autoclaved fermentation media.

The media consisted of (g/L) 2.5 grams trypticase, 250 mL buffer solution (4g$(NH_4)HC0_3$, 35g $NaHCO_3$), 250mL macro-mineral solution(5.7 g Na2HPO_4, 6.2g KH_2PO_4, 0.6g $MgSO_4.7H_20$), 125 μL micro-mineral solution (132 g $CaCl_2.2H_20$, 100g $MnCl_2.4H_20$, 10g $CoCl_2.6H_20$, 80g FeCl3.6H20) 1.25 ml, 0.1% (w/v) resazurine solution. The media was then reduced by the addition of 33.5 ml reducing solution (6.25 g cysteine hydrochloride, 6.25 g $Na_2S.9H_20$, 1.6g NaOH). pH of the resultant suspension was then adjusted to 7.2 using HCl. The micro- and macro-mineral solutions were prepared in large quantities and utilized as required. The micromineral solution was stored in a dark glass bottle and refrigerated at 4^0C to maintain the quality of the solution. The macromineral solution was also stored at 4^0C. The reducing solution was made fresh when required. Once the fresh media had been prepared for use in the fermentation experiments it was sterilized by autoclaving at 121^0C for 20 minutes and then kept at 4^0C. Tubes containing the substrate and fermentation medium were reduced for 4 hours within the anaerobic cabinet prior to inoculation with the faecal inoculum.

Frozen faecal material from individual participants (which had been mixed 1:1 with sterile anaerobic 50% glycerol (w/v), was thawed and a 10% w/v faecal slurry was prepared by homogenisation and dilution in pre-reduced PBS (0.1M, pH 7.2). The use of frozen samples compared to fresh samples in fermentation experiments has been previously validated (Rose, Venema et al. 2010). Working within an anaerobic chamber 1mL of the 10% w/v faecal slurry was added to each of the fermentation tubes (1% w/v). Tubes were capped, sealed, and incubated under anaerobic conditions with agitation for 24 hours. Constant agitation ensured that the starches, being insoluble were dispersed throughout the fermentation tube and did not form a sediment which would be unavailable for fermentation. Negative controls comprised of faecal inoculums with no added substrate and were incubated in parallel with incubations containing the

HAMS, Lactulose or mHAMS. Samples were taken at 0 h and 24 h and stored at -80^{0}C for measurement of SCFA, pH, and DNA extraction to determine microbial composition. Molecular analysis of the samples will be reported in Chapter 4.

SCFA concentration was determined using capillary column gas chromatography, which is a similar method to that described by McOrist et al (McOrist, Abell et al. 2008). Samples containing 3 x volume for weight of 1.68 mM heptanoic acid were mixed and centrifuged at 3,000 rpm for 10 minutes at 5^{o}C. The supernatant, 300 μL, with 10μL of 1M phosporic acid was then placed into filter tubes for further cleanup. Samples were subsequently loaded onto the Agilent 6890N Network gas chromatograph system with automatic loader/injector. The GC column was a Zebron ZB-FFAP (Phenomenex, Lane Cove, NSW, Australia), length 30 m, internal diameter 0.53 mm, film thickness 1 μmeter. The GC was programmed to achieve the following run parameters: initial temperature 90^{0}C, hold 0.5 min, ramp 20^{0}C/min final temperature 190^{0}C, total run time 8.0 min. Gas flow 7.7 mL/min splitless to maintain 3.26 psi column head pressure, septum purge 2.0 mL/min.

Calibration standards (Sigma-Aldrich) were prepared to give a mixture of the following concentrations of acids (mM): 26.22 acetic, 19.86 propionic, 3.24 isobutryic, 16.32 butyric, 5.40 isovaleric, 5.6 valeric, 4.74 caproic and 5.04 heptanoic. This standard mix (0.2 μlitres) was used to calculate retention times and create a standard plot. Results were expressed as μmol/g of sample. A digital pH meter was used to measure pH. Fermentation of the substrate was assumed to have occurred if there was a statistically significant decrease in pH and an increase in production of SCFA at 24 hours in ferments containing the RS when compared to negative controls.

3.3.7 Statistical Analysis

Data normality was first assessed using the Shapiro-Wilk test (conducted using SPSS EXPLORE procedure). A boxplot of the dataset was used to identify outliers (values >1.5 times the inter-quartile range). Within pre-weaning and weaning groups, paired t tests were performed to identify statistically significant differences between outcomes for each substrate. Repeated measures ANOVA was used to determine if there was a statistically significant difference between the means for the negative control and test substrate (HAMS) within the pre-weaning and weaning groups. Univariate ANOVA was used to analyse for differences in starting pH and total SCFA of the different groups within the weaning and pre-weaning infants. Due to differences in the number of formula and breast fed infants, a general linear mixed model was used to determine if within the pre-weaning group, the method of feeding influenced the effect of incubation with HAMS on both change in pH and total SCFA production.

3.4 Results

Participant characteristics are provided in Table 3-1. In all, 17 participants were studied. Only 7 mixed fed infants were recruited and of these one participant was unable to provide a weaning specimen. Mothers and infants met all the inclusion and exclusion criteria. Due to the insufficient amount of faecal material from the pre-weaning infants, fermentation of the mHAMS (Crispfilm) was unable to be assessed within this group.

Table 3-1 Description of infants used as faecal donors.

Participants	Pre-weaning (N)	Weaning (N)	Age pre-weaning sample (months)	Age at weaning sample (months)
Breast-Fed	10	10	3.32±.37	7.03±.27
Mixed-Fed*	7	6	3.29±.22	7.39±.21
Total	17	16	3.31 ± .23	7.16±.18

***received at least two formula feeds per week over a minimum period of three weeks

As per the Shapiro-Wilk test, none of the variables were sufficiently non-normal to compromise subsequent analysis. Regarding outliers, one participant was noted to have an outlier for total SCFAs for each substrate, including incubations with the negative control. This subject was excluded from further analysis of the SCFA data. A parallel series of calculations performed with the inclusion of this participant's data, did not alter the final statistical conclusions.

Results for initial, final and change in pH at 24 hours following incubation of the infant faecal microbiota with the test substrates are presented in Table 3-2. The results demonstrate that in all of the incubations, there was a significant decrease in pH at 24 hours. In both the pre-weaning and weaning groups the greatest decrease in pH occurred following incubation with lactulose. In the pre and weaning groups the decrease in pH was significantly greater following incubation with HAMS than in the negative controls, suggesting fermentation of the HAMS. In the weaning group, incubation with the mHAMS also led to a decrease in pH when compared to the negative control.

Table 3-2 Initial and final change in pH at 24 hours following incubation of infant faecal microbiota with HAMS and other substrates

	Pre-weaning			Weaning			
	Negative Control	**HAMS**	**Lactulose**	**Negative Control**	**HAMS**	**mHAMS**	**Lactulose**
Initial pH (0 h)*	7.64±.03[a]	7.63±.03[a]	7.69±.03[a]	7.66±.03[a]	7.65±.02[a]	7.65±.03[a]	7.72±.04[a]
Final pH (24 h)**	6.72 ± .08[a]	6.40 ± .09[b]	4.73 ± .12[c]	6.77± .05[a]	6.43± .05[b]	6.37± .04[b]	4.65±.08[c]
Change in pH**	-.93± .07[a]	-1.21 ± .06[b]	-3.03±.06[*c]	-.82±.07[a]	-1.14±.08[b]	-1.09±.10[b]	-2.89±.20[c]

*****Within the pre-weaning and weaning groups a one-way ANOVA was performed to compare differences in initial pH for each substrate. There was no significant difference between any of the substrates within these two groups (p>.05).
**Within the pre-weaning and weaning groups a one-way ANOVA found a significant difference in change in pH between the substrates. Bonferroni multiple comparison procedure was used to compare group means. Unlike superscript letters are significantly different (Bonferroni adjusted P< 0.05) within each weaning stage.

Analysis of variance conducted over all 7 groups showed no main effect of any of the substrates on initial pH, $F(6, 1.16)=1.136$, $p=0.36$. A mixed factor ANOVA revealed a main effect of the substrate (HAMS vs negative control) $F(1,31)=86.70$, $p<0.001$ but not the stage of weaning on the change in pH, $F(1,31)=.957$, $p=.335$. There was no interaction between substrate (HAMS or negative control) and stage of weaning $F(1,31)=.390$, $p=.537$. This indicates that the introduction to solids did not influence the effect of incubation with HAMS on the change in pH when compared to negative controls.

Within the pre-weaning group, due to differences in group size, a linear mixed model analysis was performed to look at the interaction between change in pH, substrate (HAMS or negative control) and mode of feeding. Substrate and diet were assigned as random variables; subject was assigned as a fixed factor. Analysis revealed no interaction between mode of feeding and change in pH at 24 hours of incubation with HAMS vs negative control ($p=.809$).

Table 3-3. SCFA production after 24 hrs incubation with test substrates using pre and weaning infant faecal inoculum.

	Pre-weaning			Weaning			
	Negative Control	**HAMS**	**Lactulose**	**Negative Control**	**HAMS**	**Modified HAMS**	**Lactulose**
Total SCFA (mmol/L)*#	16.68±1.7[a]	23.70±1.67[b]	61.84±5.42[c]	20.11±2.30[a]	34.85±4.04[b]	37.81±4.16[b]	78.27±4.51[c]
Acetate*	14.70±1.50[a]	20.93±1.51[b]	56.11±6.50[c]	16.98±2.02[a]	27.73±3.23[b]	30.27±3.06[b]	73.42±4.99[c]
Propionate*	.83±.17[a]	1.33±.33[a]	1.13±.80[a]	1.60±.32[a]	4.14±.91[b]	5.56±1.21[c]	2.89±.74[a,b,c]
Butyrate*	.36±.13[a]	.74±.26[b]	.27±.12[a]	1.09±.26[a]	2.20±.59[b]	1.23±.33[a]	1.81±1.04[a]
Molar ratio**	93:5:2	91:6:3	98:1:1	86:8:6	82:11:7	83:13:4	93:3:4

[#]Total SCFA = sum of acetate, proprionate, butyrate and branched SCFAs (valeric, caproic, isobutyric, isovaleric)
*Paired t tests conducted within the pre-weaning and weaning groups, values which do not share a common superscript letter are significantly different (Bonferroni corrected p<.05)
**Molar ratio: Acetate:Propionate:Butyrate

The production of short chain fatty acids was measured in each of the fermentations after 24 hours of incubation with each test substrate. In order to measure total SCFA production, in addition to acetate, butyrate and propionate, the production of the minor short chain fatty acids valeric, caproic and iso-butyric were also totalled. The means and standard error of the means for total and individual production of SCFAs at 24 hours are shown in Table 3-3.

Within the pre-weaning and weaning groups, SCFA concentrations were compared between the substrates using paired-t tests to determine significant differences. Due to multiple comparisons, Bonferroni adjustment to level of significance was made. In both groups, as expected, incubation with the positive control, lactulose, led to a significant increase in total SCFAs when compared to the other substrates. In the pre-weaning group there was a significant difference in production of SCFAs between the HAMS and the negative controls, indicating that the pre-weaning faecal inocula had some capacity to utilise HAMS as a substrate. In the weaning group there was also a significant difference in the production of SCFAs between the negative control and the two resistant starches (HAMS and mHAMS). This confirmed the fermentation of the resistant starches by the faecal inocula. In the weaning group there was no significant difference between incubation with HAMS when compared to the acetylated mHAMS.

A two-way repeated measures factorial ANOVA was performed to examine the effect of introducing solids on the production of SCFAs at 24 hours of incubation with either the negative control or HAMS. This demonstrated a statistically significant ordinal interaction between incubation with HAMS and stage of weaning, $F(1,31)=7.53$, $p=0.015$, see Fig 3-1 . This suggests that the fermentation of HAMS is enhanced post the introduction of solids. To examine simple main effects,a pairwise comparison with Bonferroni correction was performed. This revealed no statistically significant simple main effect for weaning, $p=0.06$, but did reveal a statistically significant simple main effect for substrate, $p<0.001$.

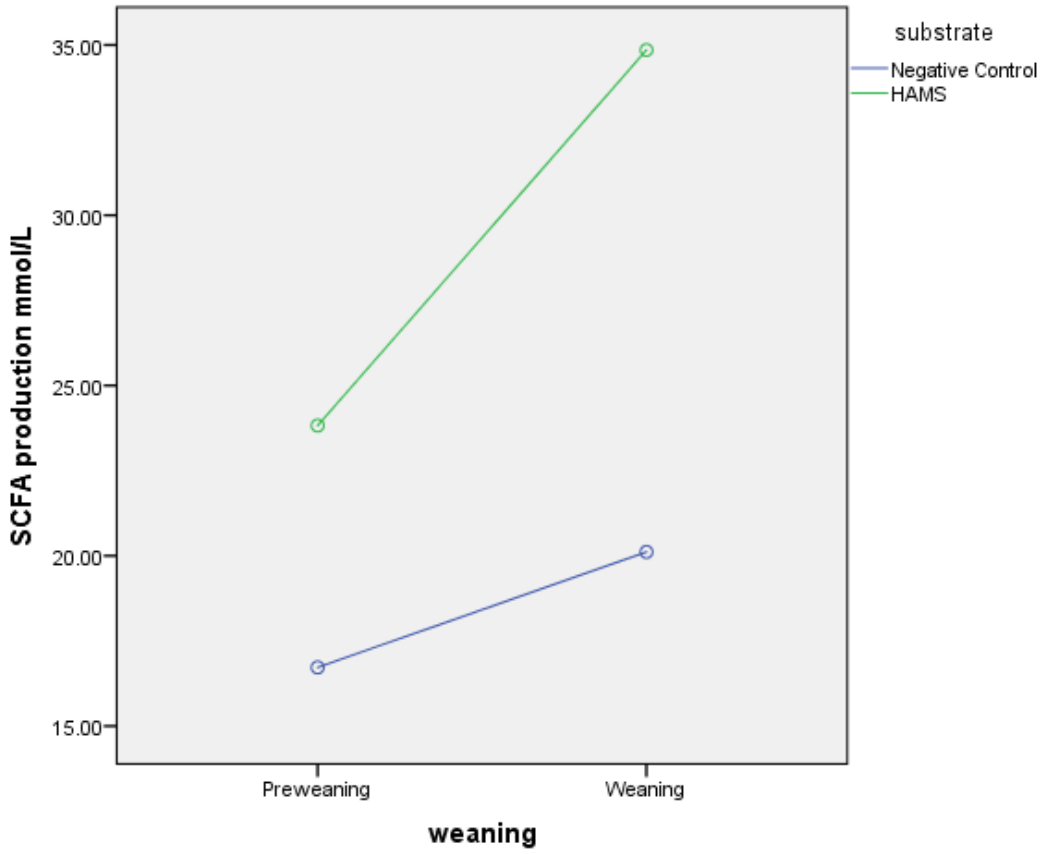


Figure 3-1 SCFA production at 24 hours in pre-weaning and weaning infants following incubation of faecal inocula with HAMS or Negative Control

Within the pre-weaning group, linear mixed model analysis revealed no effect of mode of feeding (breast or mixed-fed) on the effect of incubation with HAMS on SCFA production when compared to negative controls ($p=.754$). The molar ratios for all the substrates confirmed previous findings that in young infants, acetate is by far the dominant SCFA during fermentation. A trend for an increase in the molar ratios of propionate and butyrate with weaning was noted for all substrates. This likely reflects the major changes that occur to gut microbial composition and function, post commencement of solids.

3.5 Discussion

Dietary fibres pass through the upper intestine and are the primary substrate for fermentation by gut bacteria. Their intake is associated with the production of fermentation metabolites such as SCFAs, which have a range of beneficial effects including fostering the healthy development of the immune system (West, Renz et al. 2015). Observational studies suggest that the high fat/low fibre Westernized diet may be associated with the increased prevalence of non-communicable diseases, conditions which are invariably associated with an aberrant immune function. A major divergence in the measured intake of fibre between those living in Western countries and those consuming a predominantly agrarian diet, as occurs in many rural parts of the world, emerges as soon as solids are introduced into the diet. Thus, manipulation of the fibre content of the diet during this critical period may translate to long-term consequences for the host. It is within this context that I was interested in whether forms of resistant starch, a dietary fibre, could have a role in early childhood.

I used batch *in vitro* fermentation methodology to examine the capacity of faecal inocula from pre-weaning and early- to mid-weaning infants, to ferment two particular forms of dietary fibre, high amylose maize and modified high amylose maize starch. These resistant starches were specifically chosen due to their demonstrated health benefits in older children and adults and as bifidobacterium, an abundant group of bacteria in the faecal inocula of young infants which are thought to be particularly adept at utilising it as a substrate.

In both the weaning and pre-weaning groups, the production of SCFAs by the faecal inocula was significantly greater in the presence of HAMS when compared to negative controls (a measure of the background endogenous fermentation). This suggests that the microbiota of young infants possesses the capacity to utilise HAMS as a substrate prior to the introduction of solids. While fermentation might not be unexpected in the weaning cohort, it is somewhat surprising that the microbiota of pre-weaning infants appears to already have some capacity to ferment starch. Whether this will translate to any discernible effect on microbial composition is not yet known, and will be pursued in the following chapter.

The capacity to ferment HAMS increased post commencement of complementary feeds, as evidenced by the increase in production of SCFAs following incubation with HAMS in the weaning infants when compared to their respective negative controls. It is likely that following the introduction of solids, more diet-derived starch will arrive into the colon, increasing the amylolytic capacity of the resident bacteria even further through adaptive changes in microbial composition and function (Christian, Edwards et al. 2003).

The capacity for the faecal inocula of young infants to ferment a modified HAMS(mHAMS), starch acetate, was also examined. Crispfilm, the commercial starch acetate that was used in this study has a HAMS backbone which has been esterified with acetyl groups. Due to the limited quantity of faeces available from the pre-weaning infants, fermentation of the mHAMS was only studied in the weaning samples. Contrary to expectations, there was no significant difference in the production of total SCFAs, especially acetate, between HAMS and mHAMS despite the addition of the esterified acetate to the latter. It might have been that the degree of substitution was insufficient for cleavage of the esterified acetate to achieve a statistically significant increase in the overall pool of acetate within the fermentation tube. Future studies could consider using experimental acetylated starches with higher percentage acetylation. The potential role of such starches in adult health is being currently explored (Topping 2016).

Although this is the first such study to examine the capacity of infant faecal inocula to ferment HAMS and mHAMS, others have used *in vitro* fermentation methods to examine carbohydrate fermentation by the colonic flora of infants. Parrett et al studied the fermentation of soybean polysaccharide, a complex carbohydrate, in pre-weaning breast- and formula-fed infants (Parrett, Edwards et al. 1997). They found that, in both groups of pre-weaning infants, the production of SCFAs following incubation of the faeces with t soybean polyscaccharides was not significantly different to incubations with no added substrate. Thus, they concluded that the infants' faecal microbes, a likely representation of their colonic flora, had poor capacity to ferment soybean polysaccharide. They extrapolated this finding to suggest that the faecal inocula of early weaning infants may not have the capacity to ferment a broad range of complex carbohydrates.

However, in a later *in vitro* study of fermentation capacity, Scheiwiller et al (2006), found that weaning infants could ferment a type 3 resistant starch, as measured by an increase in SCFAs compared to negative controls and degradation of the substrate (Scheiwiller, Arrigoni et al. 2006). The weaning infant cohort in Scheiwiller's study was significantly older than those in Parrett's study (11 versus 7 months) and this could have contributed to the emergence of fermentation capacity for a complex carbohydrate. Interestingly, the fermentation capacity of Scheiwiller's older infants for the RS3 was still significantly less than that of adults (Scheiwiller, Arrigoni et al. 2006) .

However, another study by Christian et al, using raw and cooked waxy maize starch (high amylopectin:amylose ratio), found that the faecal flora of weaning infants was actually highly efficient at fermenting the starch, even more so than in adults (Christian, Edwards et al. 2003). This suggests that the structure of the complex carbohydrate is a major factor in determining whether infant faecal inocula are capable of its fermentation. Indeed, it has been shown that the amylose:amylopectin ratio and the addition of different chemical groups, can affect the capacity of bacteria to ferment the starch (Morita, Kasaoka et al. 2005).

This lack of universal consensus regarding the capacity of infant faecal inocula to ferment complex carbohydrates, justifies the need to specifically confirm the fermentation capacity of infant faecal inocula for modified and unmodified HAMS (Morita, Kasaoka et al. 2005). It is also apparent when reviewing and comparing the results from the different *in vitro* fermentation studies, that there exists no standardised methodology when performing batch *in vitro* ferments. While the development of such a method was attempted by Barry et al, the absence of molecular results in their study limits its current applicability (Barry, Hoebler et al. 1995). In addition to whether pre-digestion of the substrate is or is not performed, there are often differences between studies in relation to faecal inoculation size, initial pH, time points for the actual fermentation, and the use of pooled or non-pooled faecal inocula.

In this study, I chose a relatively small inoculum size (10 g per litre final proportion).]This was due to the small quantity of stool available from such young infants in a single collection and our system being non pH controlled. A large inoculum size has been shown to result in higher fermentation products, which could contribute to a highly acidic environment and compromise microbial viability (Long, Xue et al. 2015). While in larger inoculates there might be no need to add components to the culture medium such as micronutrients, given the small size of my inoculums, micro- and macro-nutrients were added to the fermentation media.

Similar to other infant batch fermentation studies, I chose a 24 hour time frame over which to analyse fermentation. This decision was influenced by several factors. Although certain dietary components can reach the large bowel within a few hours after ingestion, a further passage of time is required for the induction of appropriate microbial enzyme responses and/or for the microbes to reach sufficient numbers to produce physiologically relevant levels of SCFAs. If a substrate is novel to the microbial community, it might be expected that the time taken to generate a measureable fermentation response maybe longer due to the need for adaptation to the new energy source. Using a non-pH controlled system, running the *in vitro* cultures longer than 24 hours might have been likely to encounter problems with product inhibition, compromising the accuracy of future microbial analysis. Due to the limited quantity of stool available from the infants, particularly during pre-weaning, I was unable to plot a time course for the different substrates. The limited availability of faecal inocula also precluded comparative evaluation of the fermentation of the chosen resistant starch to other substrates which may already be recognised, and indeed used, as prebiotics in infants, i.e. fructo-oligosaccharides.

I also elected not to pool faeces from the different infants prior to inoculation. It has previously been suggested that individual variation in the microbiota may often exceed the effects of treatment, thus making it more difficult to establish the actual effect of certain prebiotic supplementation. In demonstrating fermentation of the test substrates, despite not pooling the faecal samples, the case to consider an *in vivo* study is perhaps strengthened.

Finally, the measured initial pH in all of the test groups was higher than that often used in batch fermentation studies. The resistance of bifidobacteria to acidic pH is poor, with the exception of *B.animalis* (Ruiz, Ruas-Madiedo et al. 2011) Indeed, the optimum growth rate for many of the human strains of bifidobacteria is a pH of around 6-7 (Ruiz, Ruas-Madiedo et al. 2011). As I was particularly interested in the growth and activity of bifidobacteria, I considered the higher initial starting pH to be tolerable. The higher initial pH may however have affected fermentation kinetics and the end products of fermentation. For instance, it is known that *in vitro* production of butyrate is unfavourable at higher pH. However, it is also known that the production of butyrate by infant faecal inocula is limited (Sellitto, Bai et al. 2012).

The early dietary habits of an infant and its impact on their emerging microbiota might also affect the predictability of the adult gut microbial response to future dietary substrates. It is well recognised that in adult intervention studies, there is significant inter individual variation in both the initial capacity to ferment high amylose maize starch (Martinez, Kim et al. 2010) and the taxonomic changes associated with its fermentation. This individual variation in the predicted response to a HAMS intervention is a concern when exploring the beneficial therapeutic and preventative health aspects of an RS intervention in adults. In addition to strain specific differences in starch fermentation and host associated factors, one reason for the differences in response might be related to variations in the earlier colonisation of the gut. Exposure to and sampling of microbial antigens during infancy, can influence future tolerance of the adult immune system to infant acquired microbiota (Ouwehand, Isolauri et al. 2002). Thus manipulating the exposure to HAMS in early childhood might not only manage the emerging ecology of the intestinal microbiota but also influence the future intensity and durability of the response to HAMS re-exposure in later life. This emerging paradigm that a future clinical response to a nutritional intervention may be dependent upon microbial composition in early childhood should be a focus for future research.

Manipulation of the colonic luminal environment through the administration of a fermentable substrate may also have effects on the absorption of minerals. Despite the majority of SCFAs being rapidly absorbed across the colonocyte, the increase in their production following fermentation of a substrate, can result in a significant reduction in luminal pH. For certain minerals, such as calcium, a decrease in pH and a resultant increase in mineral solubility, could lead to a clinically significant increase in colonic absorption. The clinical consequences of facilitating the increased absorption of divalent cations such as calcium and possibly even iron, from the colon of a growing infant are not well understood. In weanling rats, intake of modified starches has been associated with an increased incidence of nephrocalcinosis. In determining the safety of modified starches in human foods, international food authorities have concluded that the rat may be a particularly sensitive species for nephrocalcinosis and that these findings may have little relevance

to humans (JEFCA 1982a). Nevertheless, international and local guidelines still stipulate that the degree of acylation for modified food starch should not exceed 2.5%. Certainly, there is experimental evidence that increasing the degree of acylation can result in increased colonic luminal concentrations of the acylated chemical moiety. Depending on the magnitude of this increase, this may have unexpected effects on epithelial cell integrity, microbial composition and mineral absorption .

Conversely, the heightened awareness of the importance of SCFAs in determining aspects of human health may eventually result in modified starches with higher degrees of acylation being specifically used to deliver more SCFAs directly into a young child's colon. However, the potential for harm must always be considered and necessitates an incremental approach to study design.

Being an *in vitro* model this study has inherent limitations. In a small scale-batch fermentation system, as was used in this study, a steady state in microbial composition or function is not achievable. There is also uncertainty as to whether the bacterial composition of faeces accurately represents the complex microbial community within more proximal segments of the colon, where the majority of fermentation occurs. Finally, the degradation of the substrate in each ferment was not measured. However, it is not uncommon for *in vitro* fermentation studies to omit this measurement, instead relying as I have done, on the products of fermentation and changes in microbial composition to provide an estimate of fermentation.

3.6 Conclusion

The results of this *in vitro* fermentation study demonstrate that the faecal inocula of pre-weaning and early to mid-weaning infants do have the capacity to ferment HAMS and acetylated HAMS. Although such evidence is promising when considering a role for these starches in an early nutrition intervention, further analysis of the molecular changes associated with their fermentation is needed to confirm a potential prebiotic effect.

Chapter 4 Molecular changes associated with fermentation of RS by infant faecal inocula

4.1 Abstract

Background/Aims: Resistant starch (RS) has a prebiotic effect in adults. It can increase short chain fatty acid (SCFA) production and change the composition and diversity of the gut microbiota. In Chapter 3, it was shown that that the faecal inocula of weaning infants and to a lesser extent pre-weaning infants, have the capacity to ferment resistant starch in the form of high amylose maize starch. However, as the initial composition of the gut microbiota of infants differs from adults, it cannot be assumed that the compositional changes associated with the consumption of HAMS in adults will extend to infants. The aim of this study is to use an *in vitro* fermentation model to examine the changes to the microbial composition of infant faecal inocula following incubation with high amylose maize and a modified high amylose maize starch, mHAMS.

Methods: Faecal samples were collected from infants before and within 12 weeks of commencing first non-milk food. Using a 24 hr in vitro batch fermentation system, the faecal inocula from each infant was incubated with high amylose maize starch. Weaning infant inocula was also incubated with a modified high amylose maize starch. A negative control comprised faecal inocula incubated without either resistant starch. DNA was extracted from ferments at 0 and 24 hours. Illumina MiSeq sequencing and qPCR was performed on extracted DNA.

Results: In weaning infants (7.16±18 months), the fermentation of HAMS and mHAMS increased diversity (Shannon $p<0.05$) and had positive effects on the microbial profile, increasing the abundance of Bifidobacteria and Bacteroides and increasing the ratio of Bacteroidetes to Firmicutes. The incubation of HAMS with the faecal inocula of pre-weaning infants did not demonstrate similar changes.

Conclusion: The beneficial effects on microbial diversity and composition following incubation of weaning infant faecal inocula with HAMS and mHAMS suggest that these starches may function as a novel prebiotic during infancy. .

4.2 Introduction

Over the last few years there has been increased appreciation of the importance of the human gut microbiota in dictating many aspects of human health. It is now recognised that the compositional disruption of the infant's emerging gut microbiota with its subsequent adverse effects on microbial metabolic function, maybe linked to the development of various chronic diseases in the human host (West, Renz et al. 2015). Thus in order to improve human health, it may be prudent to selectively programme the gut microbiota at critical periods in its development. The initiation of solid foods represents a dynamic period of change in the composition of gut microbiota and as such the administration of prebiotics during this particular time period could have profound health consequences (Dogra, Sakwinska et al. 2015). Prebiotics are non-digestible food ingredients which selectively stimulate the growth and/or activity of one or a limited number of bacterial species that already reside in the colon (Gibson 2004). In younger children, the exogenous prebiotics inulin, galacto-oligosaccharides (GOS) and fructo-oligosaccharides(FOS) have been the main substrates to be used in clinical trials (Orel and Reberšak 2016). Although scientific societies do not recommend the addition of these or indeed any, prebiotics to standard infant formula, it is a practice performed widely among commercial infant formula companies (Vandenplas, Zakharova et al. 2015). There is some evidence to support their activities, with there being general agreement that the gut microbial changes elicited by the addition of oligosaccharides to infant products, aligns the composition of formula fed infants closer to that of breast fed infants, particularly in relation to the relative abundance of bifidobacteria (Guaraldi and Salvatori 2012).

In adults, resistant starch (RS) and more particularly a form of RS known as high amylose maize starch (HAMS), has also received interest as a potential prebiotic (Zaman and Sarbini 2015). Its ease of incorporation into regular foods and relatively low costs of production in comparison to FOS and GOS, could favour HAMS as an alternative prebiotic during infancy, particularly during weaning. However, the prebiotic properties of HAMS that have been demonstrated in adults, cannot be directly extrapolated to infants. The composition and function of the infant gut microbiota differs significantly from that of adults, and, as such, further evidence as to the prebiotic effects of HAMS within this specific age group is required.

Due to the ethical and procedural difficulties associated with direct feeding studies in infants, we will use an *in vitro* fermentation model to test the prebiotic potential of the resistant starch. As we are specifically interested in the effects of RS on the infant microbiota, we will use infant human faecal matter as the microbial inoculum to perform *in vitro* batch fermentation studies.

Thus, the aim of the present study is to investigate how incubation with HAMS and mHAMS affects the composition of microbial communities originating from pre-weaning and early-mid weaning infants during a 24 hour *in vitro* fermentation experiment.

Specific Aim1: To use in vitro fermentation methodology to determine whether pre weaning and infant fecal inocula can ferment HAMS and acetylated HAMS, as determined by changes in microbial composition.

Hypothesis: Incubation of infant faecal inocula with HAMS and modified HAMS will result in changes in microbial composition when compared to negative controls.

Specific Aim 2: To determine if fermentation of HAMS and modfified HAMS by infant inocula is associated with the selective growth of organisms which are thought to benefit human health, ie bifidobacterium.

Hypothesis: Incubation of infant faecal inocula with HAMS and acetylated HAMS will selectively increase the growth of bifidobacteria.

Specific Aim 3: To determine if there exist differences in fermentation of HAMS between weaning and preweaning infant inocula in terms of capacity to selectively stimulate the growth of organisms which are thought to benefit human health.

Hypothesis: The introduction of solid food and the subsequent changes to the faecal microbiota will influence the nature of the microbial changes following incubation of the faecal material with HAMS and mHAMS.

4.3 Materials and methods

This is not a separate study to Chapter 3. The same participants and methodology as were used in chapter 3, were used for this chapter. However, the molecular analysis of the samples is the main subject of this chapter.

4.3.1 Study design and sample collection:

This study complied with National Health and Medical Research Council (Australia) guidelines relating to ethical conduct in human research and was reviewed and approved by the Southern Health Human Ethics Committee on May 23rd 2013. Written informed consent was obtained from the legally authorized representative of the infants prior to sample collection.

4.3.2 Participants

The caregivers of pre-weaning infants were recruited by several methods, including direct approach at time of delivery or through advertisements placed at both the hospital and in a local child magazine. None of the infants or their mothers received antibiotics post delivery, including between the provision of the first and second faecal sample. Other inclusion and exclusion criteria included that the infant was full term at birth with a gestational age of more than 38 weeks; at or above the 10th percentile for weight at birth; with no known cardiac, respiratory or gastrointestinal disease and no history of diabetes. None of the infants or their breast feeding mothers received probiotic supplementation prior to stool collection. The care givers decision as to when to introduce

solids was not influenced by participation in this study. It was not possible to collect detailed records of food intake in the mothers and infants during this study. The second faecal sample was collected from infants during early to mid-weaning, which was defined as within 12 weeks of giving the first non-milk food. None of the infants provided a sample within the first two weeks of commencing solids.

Stool was also collected from five healthy adult volunteers who were recruited from a university community through word of mouth. Inclusion criteria for the adults included being between the ages of 18 and 50 years, no use of antibiotics, prebiotics, probiotics or laxatives for at least three months prior to donation and no history of physician-diagnosed gastrointestinal disease/condition.

4.3.3 Intervention

Caregivers were advised that one faecal sample would be required from each infant prior to commencing solids from at least 8 weeks of age and a second faecal sample would be collected within 6-12 weeks of solids being commenced.

4.3.4 Sample collection and processing

A portable freezer was delivered to each participant's house prior to the collection day. Within 15 minutes of an infant passing a motion, stool was collected from a disposable nappy using a sterile container. This container was then placed into an airtight bag and put into the freezer which was set at -20^{0}C. Within two hours, the freezer containing the sample was transported to the laboratory where the samples were immediately processed. Working within an anaerobic cabinet containing 5% H2, 5% CO2, and 90% N2, the faecal samples were homogenized in 50% glycerol (1:1 dilution) and then stored at -80^{0}C until further analysis. The use of frozen samples compared to fresh samples in fermentation experiments has been previously validated (Rose, Venema et al. 2010)

4.3.5 Test substrates

The two resistant starches, a HAMS (Hylon VII) and a modified HAMS (Crispfilm) were obtained from Ingredion, USA, and subjected to *in vitro* pre-digestion (see Chapter 3).

4.3.6 Small scale in vitro fermentation:

For each pre-weaning infant sample, there were three groups of incubations: HAMS, Lactulose (positive control) and negative control (blank). See Fig 4-1 for an outline of the experiment design. For the samples taken early- to mid-weaning, a fourth group, mHAMS (i.e,. Crispfilm) was included due to the increased amount of stool available from a single collection. Where faecal inocula

volume was insufficient to inoculate all incubation tubes, the lowest priority was given to inoculating the 0 hour lactulose tubes. The *in vitro* fermentation method was based on the technique described by Edwards et al (Edwards, Gibson et al. 1996) and Goni et al (Goñi and Martin-Carrón 1998). See Chapter 3 for a more detailed overview of the methodology. Three 1 mL samples were taken from each fermentation tube at 0 h and 24 h following incubation. These were stored at -80^{0}C for later measurements of SCFA, pH, and molecular analysis.

4.3.7 Preparation of adult faecal inocula for comparison of initial faecal composition to infants.

In order to confirm an expected difference in initial microbial composition between infant and adult faecal samples, stool was also collected from adult volunteers. Stool collected from five adult volunteers were pooled together, prepared and stored using the same methods as for the infant stool.

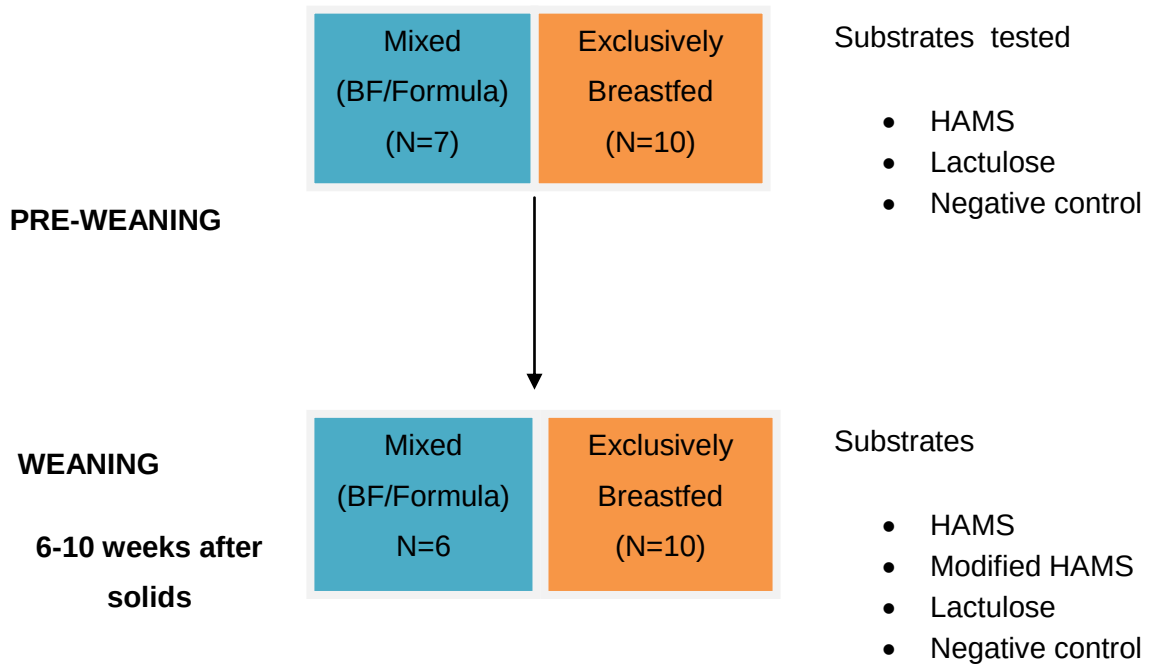


Figure 4-1 Experimental outline.

4.3.8 Molecular analysis

4.3.8.1 DNA extraction

Extraction of DNA from the ferments for subsequent quantitative real time PCR (qPCR) and sequencing was performed and analysed in general accordance with the protocol used by the major research consortium, the American Human Microbiome Project. The MoBio PowerMag Microbiome RNA/DNA isolation kit was used to extract DNA from the fermentation samples

(McInnes and Cutting 2010). This kit has been optimised for use with the EpMotion automated pipetting system (Eppendorf, France).

The major aims of the DNA extraction were firstly to maximize the yield of DNA from the samples and secondly to extract DNA of adequate quality and purity for subsequent analysis. From the trial extractions it was determined that the quantity and quality of DNA was better when we extracted DNA from 3 mL of ferment instead of 1 mL. Due to the recognised variation in fermentation between replicates during *in vitro* batch fermentation (Payne, Zihler et al. 2012), I chose to extract from a pooled sample containing 1 mL from each ferment replicate instead of taking 3 mL from a single fermentation tube. No DNA was extracted from ferments incubated with lactulose as human faecal microbiota of all age groups are recognised to readily ferment lactulose (Scheiwiller, Arrigoni et al. 2006). The purpose of incubation with the lactulose was only to confirm the viability of the faecal microbiota from the particular participant.

A sample of 1 mL from each of triplicate ferments was transferred into three separate 2mL Eppendorf tubes and centrifuged at 13,000 g at 4^0C for 5 minutes using a bench centrifuge (Eppendorf, France). Each resulting cell pellet was retained and the supernatant discarded. Heated PowerMag Microbiome lysis solution (650 µL) was added to the first of the three tubes (each containing the cell pellet) and mixed thoroughly. All the solution was then transferred into the second tube, mixed, and the contents transferred into the third tube, which was also subjected to vigorous mixing. Sterile 0.4 g PowerMag glass beads were added to the final tube and the sample was homogenized for 3 minutes at maximum speed on a Mini-Beadbeater (BioSpec Products, USA) The tube was then centrifuged for 5 minutes at 13,000 g at room temperature. The supernatant was transferred into fresh PCR grade 1.75 mL tubes. 30 µL of Proteinase K (MoBio) was added to the supernatant, mixed and kept for 10 minutes at 70^0C. 30 µL of PowerMag Inhibitor Removal Solution (MoBio) was then added to each sample and mixed well. The samples were incubated at -20^0C for 5 minutes and then were again centrifuged at 13,000 g and the supernatants transferred to fresh MoBio 2mL Deep Well Plate (Mo Bio), ensuring no transfer of any residual pellet. 5 µL of RNAse was added to each well of this Deep Well Plate (Mo Bio).

The extraction method was completed on the Eppendorf epMotion 5075TMX platform following the manufactures instructions to and using reagents from the PowerMag Microbiome DNA Isolation kit. Reagents used in this programme included Clear Mag Wash Solution, the Clear Mag Binding Solution and Clear Mag Beads. Briefly, using the automated wash and elution protocol, beads and a binding solution were added to each well and mixed together. In this process, the DNA would adhere to the beads after which the plate was transported to a magnet and the waste removed. This process was repeated 3 times with the lysate and binding solution followed by an ethanol wash. Low heat was applied to remove traces of ethanol and an elution buffer was added. This released the nucleic acids from the beads and the elute was separated from the beads and stored.

Following the extraction, the DNA was quantified using Nanodrop R ND-1000 Spectrophotometer (Wister AG, Switzerland). The presence of contaminants was identified by examining the ratio of 260nM:280nM. Where the reading was less than 1.7 DNA extraction was repeated on those samples. Where samples had a nanodrop reading of less than 30 nanomoles/uL, the readings were repeated using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scentific Inc) with the Qubit 2.0 Fluorometer. At lower DNA concentrations, fluorometric methods of DNA quantification have been shown to have greater accuracy (Gallagher and Desjardins 2001). Each sample of purified extracted DNA was diluted to a concentration of 2.8ng/μL and stored at -80°C until use. The same dilutions of DNA were used for both quantitative PCR analysis and NGS.

4.3.8.2 Real-time quantitative polymerase chain reaction (qPCR)

Selected bacteria, total bacteria, *bifidobacterium,* and *lactobacilli*, were quantified by specific primers targeting the 16S rRNA gene using quantitative real-time PCR (qPCR). See Table 4-1 for the primer sequences and the optimised qPCR conditions. The ability of a substrate to selectively stimulate the growth of a given bacterial taxon could then be determined by comparing the results of the incubations with either HAMS or mHAMS to the 24 h negative control incubations.

All qPCR analysis was performed on the CFX 384TM real-time PCR detection system (Bio-Rad, Hercules). Reactions were performed in triplicate, with a total reaction volume of 10 μL. Each reaction consisted of 3 μL (2.8 ng/μL) of DNA template and 7 μL PCR mixture containing 5uL of SYBR mix, bovine serum albumin (.2 μL), forward (.1 μL) and reverse primers (.1 μL) and PCR grade water (1.6 μL). The qPCR cycling conditions had an initial hot start at 98^0C for 3 min, followed by 35 cycles of two step qPCR with denaturing at 98^0C for 15 sec, using the annealing/elongation temperatures as shown in Table 4-1. Fluorescence intensities were detected during the last step of each cycle. qPCR melting curves were obtained after amplification by continuously collecting fluorescence intensity measurements as the reactions were slowly heated from 55 to 95^0C in increments of 0.50^0C/s. This was to verify the specificity of the amplification.

Table 4-1 Real time PCR primers used and their amplification conditions

Target	Primers	Sequence(5'-3')	Conc (nM)	Annealing		Reference
				Temp (^{0}C)	Time (Sec)	
Total Bacteria	UnivF	TCCTACGGGAGGCAGCAGT	500	60	45	(Nadkarni, Martin et al. 2002)
	UnivR	GGACTACCAGGGTATCTATCCTGTT				
Lactobacillus Spp	Lacto-F	AGCAGTAGGGAATCTTCCA	500	56	20	(Walter, Hertel et al. 2001; Heilig, Zoetendal et al. 2002)
	Lacto-R	CACCGCTACACATGGAG				
Bifidobacterium spp	Bifi –F	TCGCGTCCGGTGTGAAAG	500	56	20	(Rinttila, Kassinen et al. 2004)
	Bifi-R	CCACATCCAGCGTCCAC				

4.3.8.3 Sample derived standards for qPCR

In order to estimate the absolute abundance of the target groups in each sample, a series of sample-derived standards were created and run in parallel with the qPCR for the fermentation samples. To isolate sample-derived pure amplicons for use as standards, PCR reactions targeting the amplicons of either *bifidobacterium* spp, total bacteria or *lactobacillus* spp were performed in triplicate on three random 24 h incubation samples. The pooled amplified products were then subjected to gel electrophoresis in 1.5% agarose gels and visualised by GelGreen^{TM} staining.

The gel bands were excised and the DNA extracted. The concentration of the DNA was measured using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc). The copy number for each target per volume in the resultant pure sample was calculated as follows: (i) $\text{concentration}_{amplicon}$ (g/liter)/molecular $\text{mass}_{amplicon}$ (g/mol) = $\text{concentration}_{amplicon}$ (mol/liter);and (ii) $\text{concentration}_{amplicon}$ (mol/liter) × N_A = $\text{concentration}_{amplicon}$ (molecules/liter), where N_A is the Avogadro constant (6.022 × 10^{23} molecules/mol). The mean genome size for each target amplicon was available from the publically available resource GenBank. The sample target amplicons were serially diluted (spanning eight orders of magnitude).

Using a standard curve where the Ct value was plotted against the corresponding log of the 16S amplicon gene copy number, the starting quantity of the template for a given Ct value from each sample was calculated. This was performed using Bio-Rad CFX Manager software (version 1.5). The absolute abundance of gene targets per mL of ferment was then calculated and the log-transformed gene target counts (log10 copies/mL of ferment) were used for statistical analysis .

4.3.8.4 Sequencing of 16S ribosomal RNA encoding gene amplicons.

Culture-independent molecular methods have improved the detection of viable but non-culturable and difficult to culture bacteria, enabling a more accurate assessment of the composition of the gut microbiota. One such molecular method analyses DNA extracted from clinical samples for the presence or abundance of the small subunit ribosomal RNA gene, the 16S rRNA gene. The use of this gene for taxonomic identification is facilitated by its ubiquity across Prokaryotes and its sequence structure which includes both variable/hyper-variable and highly-conserved regions. A PCR is used to amplify a region of the 16S rRNA gene from biological samples, and by utilising next generation sequencing technology, massive parallel analysis of the DNA sequences can be performed. The information generated from sequencing is analysed and matched against databases and members of the microbial community can then be identified and their relative abundance quantified. The populations that have been inferred to exist on the basis of the sequencing data are referred to as operational taxonomic units.

In the current study, 16S ribosomal RNA (rRNA) gene sequencing was performed on DNA extracted from each participant's 24 hour fermentation samples (pre-weaning negative control, pre-weaning HAMS, weaning HAMS, weaning mHAMS, weaning negative control). An equal volume ((2 μL) of extracted DNA at a concentration of 2.8μg/mL was taken from each of the 0 time negative controls and pooled into one of 5 groups prior to 16S rRNA gene analysis (adult, pre-weaning breast-fed (BF), pre-weaning mixed-fed (MF), weaning BF, and weaning MF).

The methods outlined in Illumina's "16S Metagenomic Sequencing Library Preparation" protocol were followed (http://www.illumina.com), with minor adjustments made to PCR thermal cycle conditions, as detailed below. The hypervariable region V4 of the 16SrRNA gene was amplified from the extracted DNA using modified primer pairs with Illumina adapter overhang sequences. The full length primer sequences using standard IUPAC nucleotide nomenclature were:

16S Amplicon PCR Forward Primer =

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3'

16S Amplicon PCR Reverse Primer =

5'-TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3'

A two-step PCR process was required. The PCR reaction contained 5 μL of forward primer (1 μM), 5 μL of reverse primer (1 μM) and 12.5 μL of 2 x KAPA H-iFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA,USA) in a total volume of 25 μL. The PCR reaction was performed on a Veriti Thermal Cycler (Thermo Fisher Scientific) using the following programme: 25 cycles of 95^{o}C for 30 sec, 55^{0}C for 30 sec, 72^{o}C for 30 sec followed by holding at 72^{0}C for a further 5 min.

Following a clean-up of the PCR product, indexing PCR was then performed. This step used the Nextera XT Index Kit to attach the dual indices and the Illumina sequencing adapters. The manufacturer's instructions were followed as described in the Illumina library preparation protocol, mentioned above. The final library was pair end sequenced using a MiSeq Reagent Kit v3 on the Illumina MiSeq platform. Library preparation and sequencing was performed at Flinders University, South Australia.

4.3.8.5 Taxonomic Assignments to 16S Reads.

An in-house (CSIRO, Perth) amplicon clustering and classification pipeline based on tools from Usearch (Edgar 2010), and a RDP classifier (Wang, Garrity et al. 2007) combined with locally written tools for demultiplexing and generating OTU tables, was used to process the amplicon sequence data. The sequencing of multiple samples in a single sequencing lane was facilitated by the use of 'barcodes' – short stretches of sequence that were ligated to the start of sample sequence fragments during the library preparation. The distinct nature of these barcodes allowed for the discrimination of reads according to their sample origin. The first step in the pipeline was to perform a demultiplexing of the reads. Demultiplexing refers to the computational methods used to scan the barcode sequence pattern and bin the sequences according to the sample they belong to. Following the merging of paired reads, dereplication, clustering at 97% and chimera checking were also performed using the pipeline. Classification of the reads was then performed by both using the Ribosomal Database Project (RDP) to assign taxonomy and by also finding the closest match to the OTU from a set of reference 16S sequences. The RDP classifier, maintained at the Ribosomal Database Project, used an algorithm based on Bayes theorem to classify sequences according to Bergey's taxonomic outline (Wang, Garrity et al. 2007).

4.4 Statistical Analysis

The results for the qPCR were log10 transformed and the means were compared using a student t-test. Microbial abundance at 24 h were compared with those at 0 h for each substrate. Multivariate analysis of the sequencing data was performed using PRIMER 7 with PERMANOVA (PRIMER-E Ltd, UK). All the data were transformed with square root and a Bray Curtis dissimilarity matrix was used. Statistical analysis of Bray–Curtis dissimilarities were calculated using the relative abundance of bacterial genera following 24 hours of fermentation at the **family level**. Testing for the presence of a significant effect of sample type on beta diversity metrics was performed using permutational multivariate analysis of variance (PERMANOVA). Principal coordinate analysis (PCO) was used to visualise the dissimilarity data. This mapped the position of each sample as determined by its distance from all other points in the analysis. A p valueof <0.05 was determined to be significant.

4.5 Results

4.5.1 Verification of amplicons, primer specificity and gene-specific PCR amplification.

All qPCR assays had previously been verified using single cell colony sequencing and they were found to be 100% specific to the bacterial group assigned. Melting curve analysis revealed a single peak for bifidobacteria; however, for total bacteria and lactobacilli, the analysis revealed the presence of multiple peaks (see Appendix 8-6). This was likely due to variation in the amplicon at the region of the 16S rRNA gene. While this was an expected finding in relation to the total bacteria assay due to the great amplicon variation between bacteria, the presence of multiple peaks for lactobacilli suggested the presence of different species of lactobacilli within the faecal microbiota with variations within the analysed amplicon.

The starting abundances were calculated from a standard curve for each amplicon by plotting the starting quantity of DNA against the Ct values. This was performed using Bio-Rad CFX Manager software (version 1.5). In all assays the results were found to be linear over a range of dilutions (see Appendix 8-7). For the Bifidobacterium standard curve, the coefficient of determination (R^2) was 0.99 and the slope of the curve was -3.56, which corresponded to a PCR efficiency of 91%. For the total bacteria assay, the coefficient of determination (R^2) was 0.99 and PCR efficiency was calculated to be 93%. Finally, for the lactobacilli standard curve, the coefficient of determination was 0.99 and PCR efficiency was 92%. The absolute quantity of gene targets per mL of ferment was calculated and the log-transformed gene target counts (log10 copies/mL of ferment) were used for statistical analysis.

In the pre-weaning infants, the absolute abundance for total bacteria increased after 24 hours of fermentation in HAMS and negative controls (see Table 4-2) compared to 0 time in the negative controls (representative of the baseline). However, at 24 hours the absolute abundance of total bacteria, bifidobacteria and lactobacilli did not differ between the negative control and the HAMS incubations. In the weaning infants, following 24 hours of *in vitro* fermentation, the absolute abundance of total bacteria, bifidobacteria and lactobacilli increased in all three groups (negative control, HAMS, and mHAMS) when compared to the 0 time value in the negative controls. While there was no difference in the absolute abundance of total bacteria or lactobacilli between the three groups at 24 hours, the absolute abundance of bifidobacteria was significantly greater than in the negative controls. This indicates that both HAMS and mHAMS can stimulate the growth of bifidobacteria in the faecal microbiota of weaning infants.

The increase in total bacteria in the negative control cultures in the pre and weaning infants was likely due to the presence of residual fermentable carbohydrates naturally present in the stool. In the pre-weaning infants this was likely to include oligosaccharides from formula, and breast milk and mucins in the faecal medium. At weaning, the addition of undigested substrates from the consumption of solids was an additional contributing factor.

Table 4-2: 16S rDNA copy numbers (log 10 copy numbers mL^{-1} fermentation effluent) of specific bacterial groups before (0 h) and after 24 hr's of *in vitro* fermentation with infant faecal inocula and test substrates, as determined by qPCR

		Total bacteria		Lactobacillus		Bifidobacterium	
Pre-weaning	Substrate	0 h	24 h	0 h	24 h	0 h	24 h
	Negative control (n=17)	6.6±.15[1]	7.18±.24[a2]	4.26±.4[1]	4.19±.23[1a]	6.06±.09[1]	6.08±.32[a1]
	HAMS (n=17)		7.21±.1[a2]		4.02±.28[1a]		6.39±.25[a1]
Weaning	Negative control (n=16)	6.65±.07[1]	7.32±.06[a2]	2.9±.27[1]	3.34±.86[2a]	5.09±.31[1]	5.56±.21[a2]
	HAMS (n=16)		7.42±.06[a2]		3.52±.91[2a]		6.21±.10[b2]
	Modified HAMS (n=15)		7.47±.05[a2]		3.49±.89[2a]		6.04±.19[b2]

In the pre-weaning and weaning groups, within a column values which do not share a superscript letter are significantly different ($p<0.05$). Across the rows, where superscript numbers differ between the 0 and 24 hour times for each bacterial group, they are significantly different ($p<0.05$)

4.5.2 Bacterial 16S rDNA sequencing:

Across all samples, including the samples collected at 24 h and the pooled 0 h negative control samples, there were 321 operational taxonomic units identified, with an average of 94 OTUs per sample (59-213). A total of 632,032 usable reads were obtained from the pre-weaning faecal fermentation samples for downstream analysis. The average number of reads for the negative controls was 21,896 (4,974-36,284) and the fermentations with the HAMS gave an average of 20,240 reads (13,932-25,217). For the weaning faecal fermentation samples there was a total of 873,706 reads. The average number of reads for the negative controls was 16,594 (9,474-26,864), the mHAMS 19,433 (12,204-24,503) and the HAMS 19,793 (10,772-32,920)

For the five samples comprising of pooled 0 time negative control samples, the total number of reads was 143,316 and the average number of reads per sample was 28,663 (17,865-33,162). Of the 84 ferment samples in total which were subjected to DNA extraction, two DNA samples from the pre-weaning group failed to amplify during library preparation, and therefore these participants were omitted from the sequence analysis.

4.6 Microbial composition of baseline faecal inocula

The bacterial composition of the starting faecal inocula of the four different infant feeding groups (obtained by pooling of samples as described earlier) is presented in Fig 4-2. For comparison the microbial composition of adult faecal inocula from 5 healthy donors was also examined. Data is presented at the family level. The pre-weaning infant group was divided into exclusively breast fed (n=10) and mixed formula/breast fed groups (n=6). Stool was collected from the same infants following the introduction of solids and they were maintained in their different groups at weaning so as to examine the effect of introducing solids on microbial composition. One infant in the mixed dietary group did not provide a weaning sample.

A visual difference between adults and infants in terms of initial microbial composition is apparent from Fig 4-2. In adults, the predominant family members were Ruminococcae and Lachnospiraceae. In contrast, in the pre-weaning infants, Bifidobacteriaceae was the predominant member in both the exclusively BF and mixed-fed group. Erysipelotrichaceae and Lachnospiraceae were less abundant in the breast-milk-fed infant faecal microbiotas. Following the introduction of solids, a reduction in the relative abundance of Bifidobacteriaceae and Enterobacteriaceae was evident in both feeding groups. Concomitantly, the abundance of Lachnospiraceae and Bacteroidaceae was increased in both dietary groups from their pre-weaning baseline. The composition of the weaning infants remained very different from that of the adult samples.

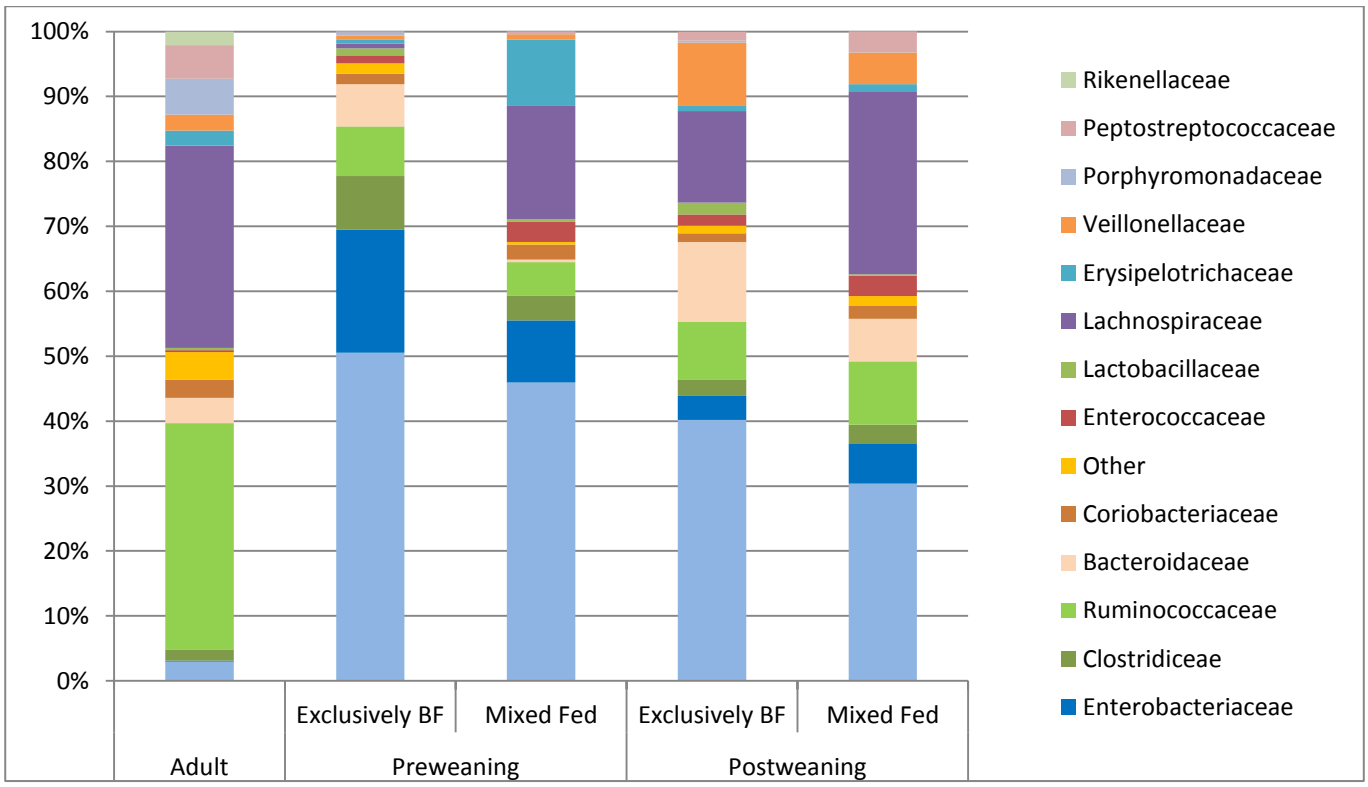


Figure 4-2 Initial composition of the microbiota from pooled negative control samples at 0 hrs at family level.

4.6.1 Changes in faecal microbial communities after 24 hours in vitro fermentation

In order to identify the changes in the composition of bacterial communities that might utilise the resistant starches, the relative abundances of bacterial groups in fermentation fluid at 24 hours of incubation was assessed. There was no pooling of the samples between individuals for the analysis at 24 hours of incubation. The relative abundance was calculated and is presented in Table 4-3. Across all levels of classification, there was a greater number of statistically significant differences in the relative abundance of organisms between HAMS and negative controls in the weaning group when compared to the pre-weaning group (see Table 4-3). At the phyla level in weaning infants, there was a significant increase in the proportion of Actinobacteria and Bacteroidetes at 24 hours of fermentation for both resistant starches in comparison to the negative controls. There was also a significant reduction in Proteobacteria following incubation with both HAMS and mHAMS.

At a genus level in the weaning infants, the abundances of Bacteroides and Bifidobacterium were significantly increased following incubation with the resistant starches when compared to the negative controls. Incubation with both HAMS and mHAMS also led to a concomitant reduction in the relative abundance of *Enterobacter.* Compared to the negative control, the relative abundance of *Ruminococcus* was significantly increased following incubation with mHAMS, but not HAMS.

Fig 4-3 illustrates the microbial composition after 24 hours of fermentation at a genus level for each substrate. Using 16S it was not possible to accurately resolve to species level, consequently genus level data only presented

Table 4-3 Percent abundance of the dominant major bacterial taxa at each taxonomic level

Taxonomy	Pre-weaning (n=15)			Weaning (n=14)				
	Control	HAMS	p value	Control	HAMS	p value	mHAMS	P value
Phylum								
Actinobacteria	12.50	15.48	0.35	10.16	18.55	**0.004**	17.88	**0.004**
Bacteroidetes	7.87	7.29	0.86	4.73	13.04	**0.001**	15.56	**0.03**
Firmicutes	33.33	37.74	0.09	33.5	32.51	0.79	34.37	0.11
Proteobacteria	46.23	39.39	0.17	51.27	35.76	**0.001**	32.06	**0.004**
Class/subclass								
Actinobacteria	12.51	15.48	0.35	10.17	18.56	**0.004**	17.88	**0.004**
Bacilli	13.46	18.74	**0.02**	14.48	16.83	0.32	19.04	0.08
Bacteroidia	7.87	7.29	0.86	4.73	13.04	**0.001**	15.56	**0.001**
Clostridia	17.71	16.28	0.58	16.25	12.31	0.26	10.79	0.07
Gammaproteobacteria	46.10	39.13	0.17	50.69	35.19	**<0.001**	31.56	**<0.001**
Order								
Bacteroidales	7.87	7.29	0.86	4.73	13.04	**0.001**	15.56	**0.001**
Bifidobacteriales	11.64	14.73	0.32	9.12	16.83	**0.01**	16.33	**0.01**
Clostridiales	17.71	16.27	0.58	16.24	12.30	0.26	10.79	0.07
Enterobacteriales	45.98	39.04	0.17	50.61	35.16	**<0.001**	31.51	**<0.001**
Erysipelotrichales	1.77	1.73	0.93	0.25	0.54	0.07	0.77	0.09
Lactobacillales	12.81	17.67	**0.03**	14.44	16.58	0.36	19.03	0.08
Family								
Bifidobacteriaceae	11.67	14.82	0.32	9.31	17.54	**0.007**	16.86	**0.004**
Bacteroidaceae	7.49	7.06	0.89	4.60	12.91	**<0.001**	14.34	**0.002**
Enterococcaceae	12.42	17.04	**0.03**	12.79	13.96	0.61	16.32	0.16
Clostridiceae	14.58	13.44	0.67	7.71	5.22	0.27	4.98	0.13
Erysipelotrichaceae	1.77	1.73	0.94	0.26	0.55	0.07	0.78	0.08
Peptostreptococcaceae	1.99	1.57	0.45	3.14	0.94	**0.04**	0.54	**0.02**
Ruminococcaceae	0.42	0.34	0.34	1.59	1.46	0.62	2.02	0.28
Enterobacteriaceae	46.12	39.19	0.17	51.19	35.88	**<0.001**	32.20	**<0.001**
Genus								
Bacteroides	7.48	7.02	0.89	4.54	12.56	**0.009**	13.92	**0.002**
Bifidobacterium	11.64	14.73	0.32	9.12	16.83	**0.006**	16.33	**0.006**
Clostridium	14.53	13.38	0.67	7.44	5.09	0.27	4.78	0.11
Enterobacter	30.31	24.89	0.21	32.32	18.21	**<0.001**	15.02	**<0.001**
Enterococcus	12.40	16.99	**0.03**	12.72	13.75	0.65	16.12	0.18
Escherichia/Shigella	15.05	13.67	0.73	16.39	15.88	0.73	15.59	0.67
Ruminococcus	0.22	0.17	0.43	0.50	0.56	0.58	1.35	**0.01**

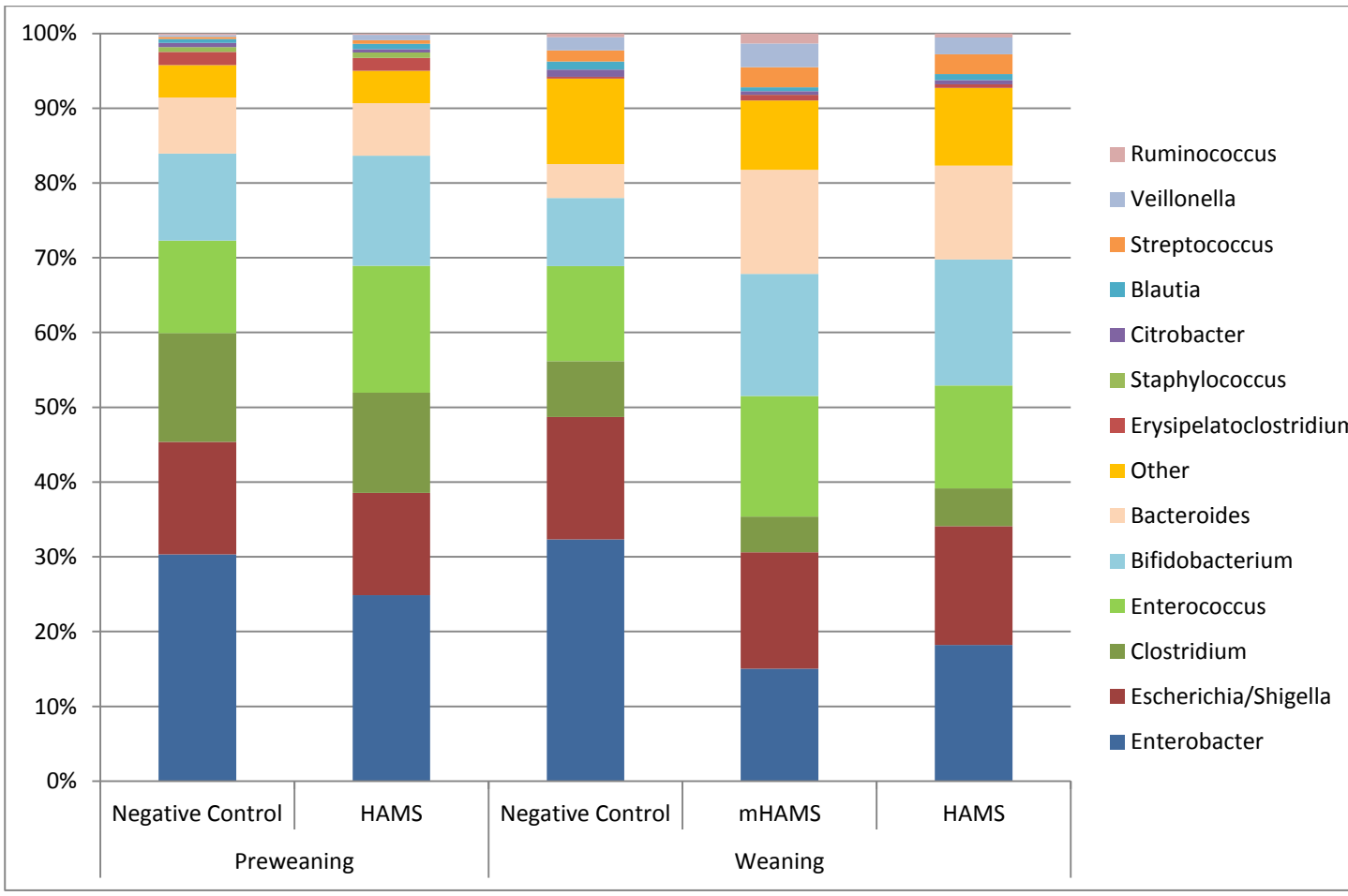


Figure 4-3 Genus-level composition of the microbial community after sequencing. DNA from 24 h *in vitro* fermentations of infant faecal inocula. Data are for 15-16 fermentations in each group.

4.6.2 Community analysis

4.6.2.1 Alpha diversity

Community diversity was assessed using the Shannon Diversity index. In the pre-weaning group at 24 hours of incubation, there was no significant difference in the Shannon Index between the bacterial communities following incubation with HAMS or the negative controls (see Appendix 8-8). This held true at all tested taxonomic levels (phylum to genus). For example, at the class level, a one way ANOVA determined that in the pre-weaning group, the overall mean of the log(e) of the Shannon Index did not differ between the negative control and the HAMS incubations ($F = 2.80, df=1, p=.10$). the Shannon Diversity Index boxplot based on OTU abundance at the class level is presented in Fig 4-4(a).

In the weaning group, at the phylum to class level, there was a significant increase in the Shannon index in the RS groups compared to the negative controls (see Appendix 8-8). For example, at the

class level in the weaning group the one way ANOVA comparing the mean of the log(e) of Shannon's Index for each substrate was highly significant (F=4.76, df=2, p=0.014). Post hoc comparison using t tests with Bonferroni correction indicated that the mean H'loge for the negative control incubations (M=1.28, SD=0.22) was significantly different than for the mHAMS (M =1.51, SD =.23, p<0.001) and HAMS (1.48, SD=.22, p<0.01) However, the type of RS (mHAMS or HAMS) did not have an effect on this measure of diversity. The Shannon Diversity Index boxplot based on OTU abundance at the class level for the weaning infants is presented in Fig 4-4(b).

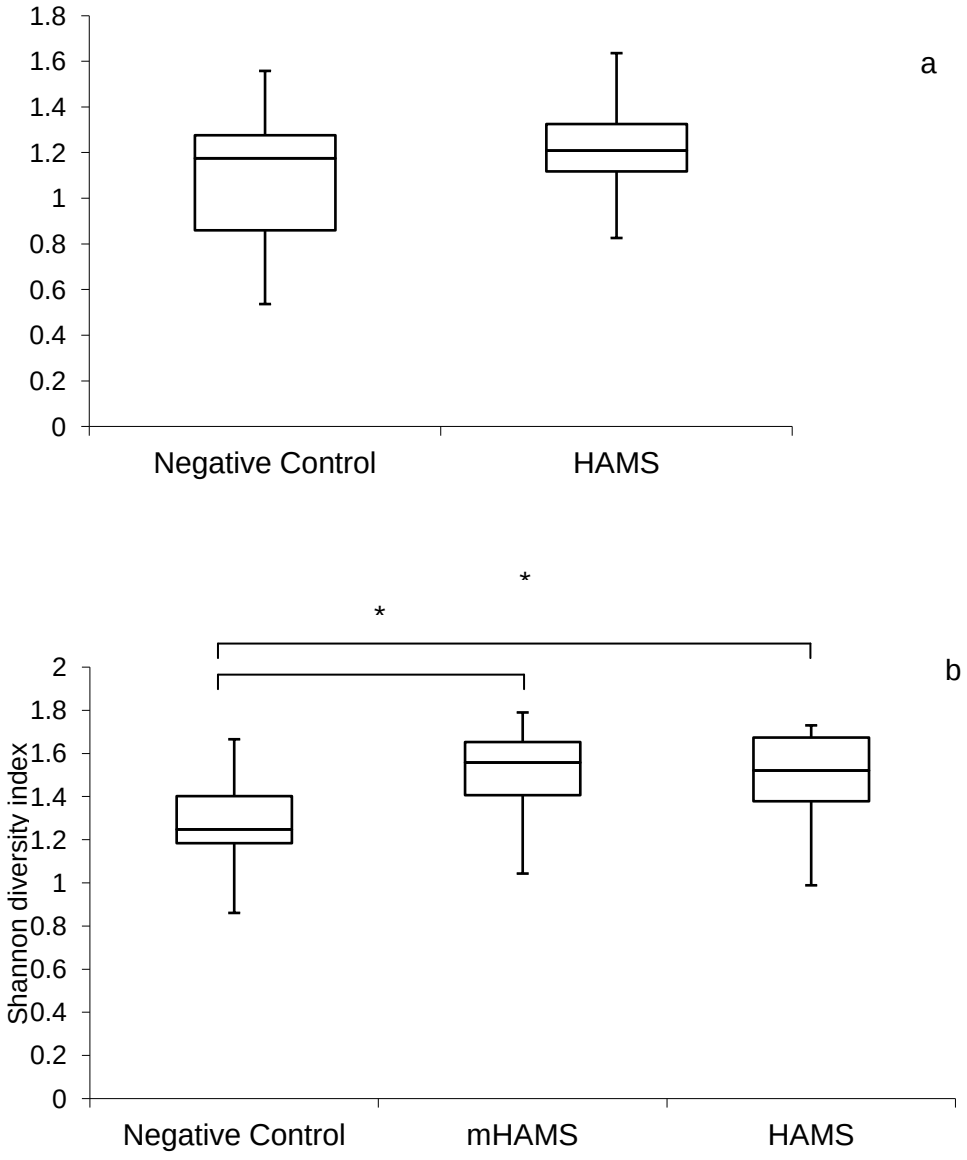
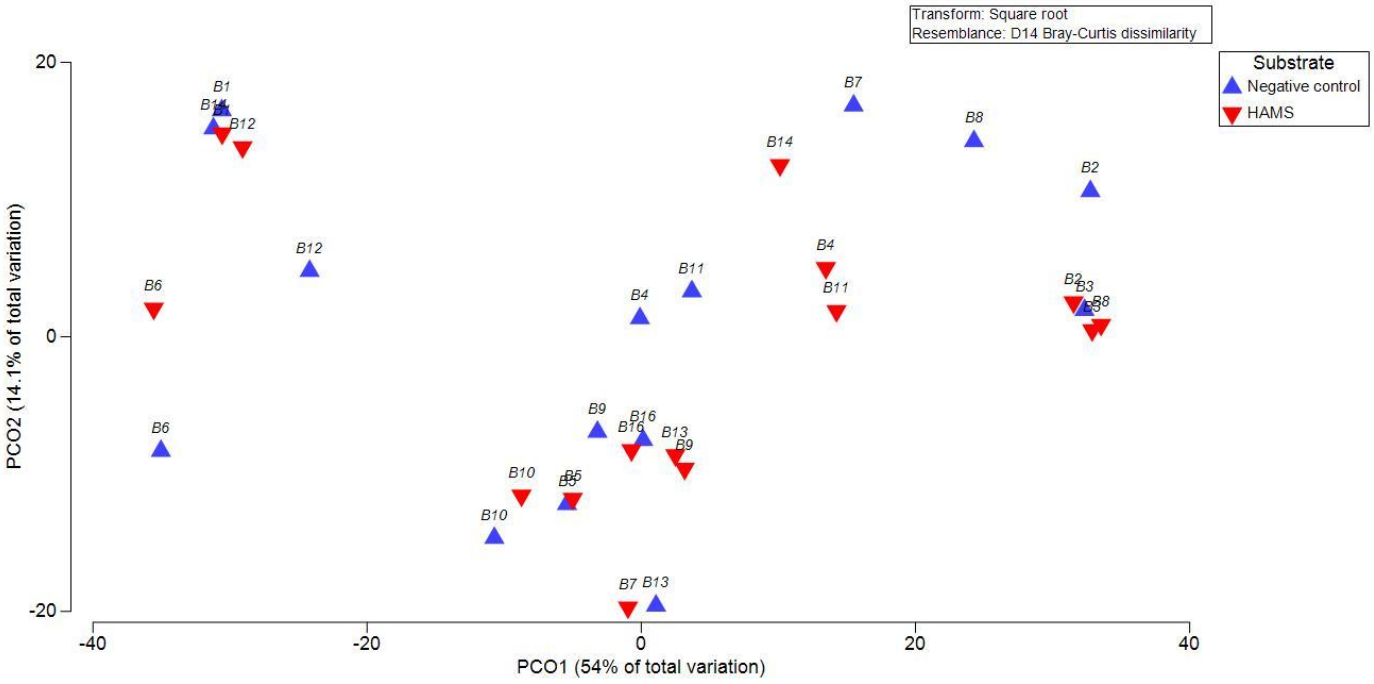


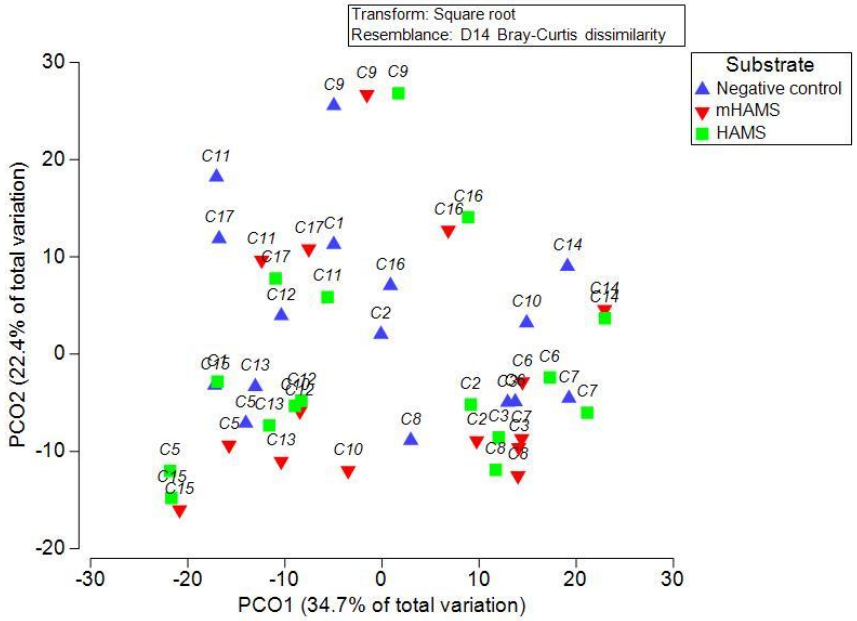
Figure 4-4 Boxplot of diversity at class level in pre-weaning (a) and weaning (b) infant fecal inocula following 24 hr in vitro fermentation. Boxes indicate 25th to 75th percentiles, with mean values marked as a line and whiskers indicating minimum and maximum values. *P<0.05

4.6.2.2 Beta diversity

Among the pre-weaning samples, multivariate analysis did not reveal an effect of HAMS on microbial community structure ($p>0.05$). However, in the weaning samples, the effect of both HAMS and mHAMS were significant ($p=0.05$), compared to the negative controls. Pairwise comparison was also performed to investigate the differences between the two test groups. This demonstrated that both resistant starches had a similar effect on community structure ($p=0.97$). Using the calculated Bray Curtis dissimilarity matrix, PCO was utilised as a method for preparing a visual interpretation of the data, see Fig 4-5. It is also apparent from Fig 4-5 that samples from the same individual cluster closely.



a



b

Figure 4-5 Principal coordinate analysis of the Bray Curtis dissimilarity matrix for (a) pre-weaning samples and (b) weaning samples, calculated at the family level.

4.6.2.3 SIMPER

Similarity percentage analysis (SIMPER) was used to demonstrate which bacterial groups were responsible for the differences between the substrates in the weaning samples. The analysis revealed that for both mHAMS and HAMS, the five groups of bacteria that were responsible for at least 58% of the difference from the negative controls were Bacteroidaceae, Enterococcaceae, Clostridiceae, Bifidobacteriaceae and Enterobacteriaceae. These bacterial groups represent an average of 82% of the classified reads in the three groups. Table 4-4 presents the results of the similarity percentage (SIMPER) analysis showing the contribution of top five family members to the dissimilarity between, firstly the mHAMS and the negative control samples and then the HAMS and negative control samples, as calculated using the Bray Curtis dissimilarity index. The mean abundance and percentage contribution of each family member to this dissimilarity is also included

Table 4-4 SIMPER analysis in weaning samples

				Negative control vs mHAMS		Negative control vs HAMS	
Family	*% Av Abundance Negative Controls*	*% Av Abundance modified HAMS (mHAMS)*	*% Av Abundance HAMS*	*Average dissimilarity ± SD*	*% Contribution*	*Average dissimilarity ± SD*	*% Contribution*
Bacteroidacee	1.86	3.19	3.02	*4.15±1.5*	12.39	3.98±1.6	12.23
Enterococcaceae	3.15	3.72	3.36	3.53±1.35	10.55	3.53±1.36	10.84
Enterobacteriaceae	7.05	5.57	5.9	3.39±1.52	10.13	3.06±1.49	9.41
Clostridiceae	2.07	1.85	1.94	3.14±1.19	9.37	3.17±1.26	9.74
Bifidobacteriacea	2.82	3.89	3.96	3.12±1.37	9.31	3.15±1.24	9.67

4.7 Discussion

Informed nutritional strategies conducted during early childhood may eventually contribute to a decrease in the myriad of immune mediated diseases that are increasingly prevalent in the developed world. The rationale underpinning such a proposal is that the dietary manipulation of the emerging early gut microbiota during a critical window period, might lead to functional and compositional changes which will benefit the host's developing immune system (Tang and Lodge 2016).

High amylose maize starch (HAMS), is a form of resistant starch that can be readily incorporated into food without altering its processing properties. In adults, the intake of HAMS and its subsequent fermentation by colonic bacteria, has a favourable effect on gut microbial composition the production of SCFAs and the host metabolic profile (Bodinham, Smith et al. 2014) (Bird, Conlon et al. 2010; Maki, Pelkman et al. 2012). The potential for infants to also benefit from a dietary HAMS intervention has not been previously considered.

Due to the ethical issues and limitations of feeding a novel substrate to human infants, I chose an *in vitro* fermentation model as a preliminary method to investigate the potential effects of HAMS, and a commercially available chemically mHAMS, on the composition of the infant gut microbiota. Inclusion of the mHAMS was due to studies having suggested that the chemical modification of a resistant starch may influence its potential prebiotic effect (Martinez, Kim et al. 2010).

The findings indicate that the microbiota of pre-weaning infants has not yet acquired the capacity to utilise HAMS as a potential substrate. However within only eight weeks of commencing solids, such a capacity emerges. If the selective stimulation of bifidobacteria and an increase in the production of SCFAs are components of the strict definition of a prebiotic (Boehm and Moro 2008), then based on these findings, HAMS and mHAMS may well function as a novel prebiotic during infancy.

Although it is well known that several *Bifidobacterium* spp are adept at the utilisation of human milk oligosaccharides (HMOs), what is less well known is that many members also possess genes that encode for enzymes involved in the fermentation of plant-derived sugars, substrates that would not be encountered until solids are first introduced into the diet (Pokusaeva, Fitzgerald et al. 2011). A significant number of *Bifidobacterium* spp possess a number of genes belonging to the GH13 family of glycosyl hydroloases (GH)(Odamaki, Horigome et al. 2015). Enzymes belonging to this family are heavily involved in the degradation of starch and starch related substrates (Van der Maarel and Leemhuis 2013).

Despite the relatively high abundance of *Bifidobacterium*, the inocula of our pre-weaning infants did not demonstrate substrate utilisation as measured by selective differences in the abundance of groups of bacteria in comparison to the negative controls. Several possibilities exist for this finding. It could be that the significant inter-individual variation in the gut microbial composition of pre-weaning infants might have masked the capacity to establish substrate level differences in the microbial profile (Avershina, Storro et al. 2014). However, another explanation rests upon differences in metabolic function amongst various members of the *Bifidobacterium* genus. Depending on the particular ecological niche, given the myriad of metabolic pathways available to Bifidobacteria spp, selective pressure will support the growth of those bifidobacteria that are able to utilise the substrates that are most available (Pokusaeva, Fitzgerald et al. 2011). Metagenomic studies have demonstrated that there are differences in which bifidobacterial glycoside hydrolase (GH) genes are transcribed between infants and adults (Milani, Lugli et al. 2015). For example in breast feeding infants, there is greater transcription of bifidobacterial GH genes that are involved in the degradation of HMO and mucin, compared to adults which reveals a greater predominance of bifidobacterial GH encoding genes involved in the breakdown of complex plant-derived carbohydrates (Milani, Lugli et al. 2015).

Following the introduction of solids, when more complex carbohydrates that are not metabolized by the digestive enzymes of the host are first encountered by the infant gut microbiota, those genes that are involved in the utilisation of these novel nutrients will be switched on (Rakoff-Nahoum, Kong et al. 2015). Given the immaturity of pancreatic function in the young infant, following the initiation of solids, significant amounts of dietary starch will enter the infant's colon. This will favour a selective increase in the expression of genes associated with starch utilisation and may account for the findings in the weaning faecal inocula.

Supporting the growth of bifidobacteria during the peri-weaning period may have far reaching consequences. The extensive saccharolytic capacity of members of the bifidobacteria genus may, through the effects of cross-feeding, support the growth of other members of the microbial community (Milani, Lugli et al. 2015). Thus, bifidobacteria may function as a scaffold upon which a more diverse microbial community could be established. The continued importance of bifidobacteria beyond the infant period should not be diminished by its low relative abundance in the healthy adult gut microbiome. As mentioned, metagenomic datasets demonstrate that the functional contribution of bifidobacteria, particularly in relation to carbohydrate digestion, is still of significance even in adulthood (Milani, Lugli et al. 2015).

Some of the purported benefits of bifidobacteria are species specific (Picard, Fioramonti et al. 2005). Traditionally, isolates displaying more than 97% 16 S rRNA gene sequence identity are considered to belong to the same species. However *Bifidobacterium* spp have a relatively high 16S rRNA gene sequence identity (Matsuki, Watanabe et al. 1999) and as such species level

identification using 16S rRNA gene alone may be problematic. Keeping in mind the limited resolution power of the 16S rRNA gene among closely-related species, we found that the Bifidobacterium genus was almost exclusively comprised of *Bifidobacterium longum*. This finding held true across all groups and across all substrates, including the negative controls and 0 time pooled samples. Although *B.longum* is certainly capable of high amylose maize starch degradation, future in vivo studies should consider additional, more discriminative gene targets for identification and quantification of which *Bifidobacterium* species are categorically stimulated by the ingestion of HAMS or mHAMS in infants.

The study also found that incubation of HAMS and mHAMS with weaning infant inocula, stimulated the growth of the phylum Bacteroidetes. According to the SIMPER analysis, Bacteroidaceae was the group most responsible for the difference in the Bray Curtis Dissimilarity Index between incubations with HAMS or mHAMS and the negative control. A decrease in the ratio of Bacteroidetes:Firmicutes in the adult gut microbiota has been associated with adverse effects such as intestinal inflammation and obesity (Turnbaugh, Ley et al. 2006). In this study we have demonstrated that in weaning infants, incubation of faecal inocula with HAMS or mHAMS reduces this ratio in comparison to the negative control. Children living in Western countries have a lower Bacteroidetes:Firmicutes ratio in comparison to children raised on more traditional plant based diets (De Filippo, Cavalieri et al. 2010). Western children also have a greater likelihood of developing obesity and certain autoimmune related inflammatory conditions (Okada, Kuhn et al. 2010). It is not yet known whether at an individual or at a population level, dietary strategies to reverse this ratio will translate into any health effects.

It has also been reported that the stimulation of particular species belonging to the Bacteroidetes family might confer health benefits, particularly in relation to mediating allergy risk in early childhood (Rodriguez, Prioult et al. 2012). Studies have shown that a lowered Bacteroides spp abundance in early life may predict the development of atopic dermatitis and food allergies (Bjorksten, Naaber et al. 1999; Bjorksten, Sepp et al. 2001). One mechanism for this may be through the promotion of CD4 T cell function by polysaccharide A, a protein which is produced by certain species of Bacteroidetes (Johnson, Jones et al. 2015) (Mazmanian, Liu et al. 2005) Consequently our finding that incubation with both HAMS and mHAMS led to an increase in the relative abundance of Bacteroidetes maybe a favourable result.

The *in vivo* effects of feeding resistant starch, either modified or unmodified, on the infant gut microbiota has never been tested. In a study feeding modified and unmodified resistant starch to adult human participants, different effects on human faecal microbiota composition between the two starches were found (Martinez, Kim et al. 2010). The authors found that the intake of the modified, but not the unmodified starch, over a three week period led to a significant increase in the abundance of Actinobacteria and Bacteroides. Using our *in vitro* model we demonstrated a

similar taxonomic effect following incubation of the weaning infant faeces with the modified resistant starch. However in contrast to Martinez et al we also found that incubation of the unmodified resistant starch with the same faecal inocula led to similar taxonomic changes. This concordance between HAMS and mHAMS in relation to the effects on microbial composition and diversity, could be due to the minimal nature of the chemical modification. The mHAMS, starch acetate, that was used in this study is approved for consumption in infant foods. For starch acetate, the FDA (Food and Drug Admission, USA) and the WHO have recommended an acetyl group's percentage below 2.5g/100g (corresponding to a maximum DS of 0.1) for a food application. Although HAMS with higher degrees of acetylation have been shown to facilitate the increased delivery of short chain fatty acids into the human colon, their comparable effects on the gut microbiota have not yet been examined (Clarke, Bird et al. 2007). Given the limited amount of infant faecal material that was available for the purposes of this study, the prebiotic effect of incubating infant faecal inocula with these experimental modified starches was not examined. From a translational viewpoint, if HAMS is to eventually be widely used in infant nutrition, the unmodified form may have greater appeal to caregivers.

It had been suggested that *Ruminococcus bromii,* a member of the Firmicutes is a keystone species in the degradation of HAMS (Ze, Duncan et al. 2012). Our results do not support such an assertion. There was no selective increase in the abundance of *R bromii* following incubation of HAMS with the weaning infant inocula. This was despite evidence of starch utilisation as seen in the increase in the relative abundance of other groups of bacteria in comparison to the negative controls. This finding, that many strains of bacteria might be able to utilize high amylose maize starch, is in agreement with several studies conducted on pigs (Jiang, Li et al. 2013; Fouhse, Gänzle et al. 2015).

Our study does have limitations. The *in vitro* environment does not accurately portray the *in vivo* environment. This is evident in the high relative abundance of Proteobacteria at 24 hours of incubation, particularly in the negative controls. Although the relative abundance of Proteobacter is known to be greater in infants than in adults, our results suggest that the *in vitro* conditions might have favoured the growth of Proteobacteria. The taxonomic representation of the different groups in the initial starting faecal inocula was however in keeping with published findings regarding human faecal microbiota composition at different stages of life (Dogra, Sakwinska et al. 2015; Jandhyala, Talukdar et al. 2015). The concordance of our findings with the available literature regarding human faecal microbiota composition, supports the methodologies that were employed to collect, preserve and store the infant stool for the fermentation experiments. The use of frozen stool with glycerol as a cryoprotective agent has already been shown to preserve the functional capacity and composition of the microbiota (Rose, Keshavarzian et al. 2009).

It must be acknowledged that the use of faecal material as a representation of the entire gut microbiota is somewhat limited, particularly as it risks under-representing those members of the microbial community which might be adherent to the colonic mucosa (Grice and Segre 2012). However, given the invasive nature of retrieving mucosal adherent members of the gut microbiota, faecal specimens will continue to be widely used as a representation of the human gut microbiota.

A further issue with batch *in vitro* fermentation is the potential for inconsistent results across laboratories (Kim, Kim et al. 2011). There is no standard method for the composition of the fermentation media, the preparation of the faecal inoculate or what percentage of faecal inoculums is transferred into the fermentation reactor. Variation in any of these parameters can lead to discrepancies in results (Walker, Duncan et al. 2005). For example, a one unit pH shift in a fermentation reactor, can cause a major change in the composition of the microbiota (Walker, Duncan et al. 2005). In our study, the initial median pH of the fermentation was slightly higher than the values reported in other comparable studies (Parrett and Edwards 1997), (Scheiwiller, Arrigoni et al. 2006).

There also exists no consensus regarding how outcomes should be measured when performing *in vitro* fermentation studies (Long, Xue et al. 2015). For instance, in order to quantify the growth of bacteria that are thought to confer beneficial effects, such as *bifidobacterium,* some researchers use a baseline control comprising microbial samples taken at an initial zero hour time point as a reference control. However, it has previously been demonstrated that even within negative control incubations, marked changes in microbial composition will occur over time (Long, Xue et al. 2015). The nature of this change will depend on several factors such as nutrient availability within the culture medium, the ability to maintain strict anaerobic conditions, and changes in the pH of the reaction. Thus, when analysing data from *in vitro* fermentation systems, it would be erroneous to rely only on the baseline controls when evaluating the actual effect of a prebiotic on microbial composition. For this purpose, the use of a 24 hour negative parallel control, as included in this study, enables the determination of what changes in microbial composition are actually due to the test substrate.

It could be argued that a change in microbiota composition during *in vitro* fermentation cannot be unequivocally attributed to the direct stimulation of growth by a particular substrate. Such growth might be due to indirect effects such as acidification of the fermentation reaction or the provision of SCFAs which, *in vivo*, might otherwise have been absorbed across the enterocyte (Gijs, Van Eunen et al. 2013). While cross feeding and luminal acidification also occurs *in vivo*, the resultant changes to the gut microbiota reflect genuine, as opposed to empirical manipulation of physiological conditions.

Despite these concerns, small scale batch *in vitro* fermentation is a widely accepted method for the initial screening of the potential of a substrate to function as a prebiotic (Harri and Päivi 2006) . For the purposes of this study, the simple batch fermentation method was chosen due to the limited amount of infant faecal inoculum available, its relative lower cost and the limited availability of more complex simulators. It also avoids direct feeding to infants, which is not appropriate prior to weaning.

Ultimately, any claim to prebiotic status for HAMS during the infant period must be supported by well designed human studies. However, direct feeding studies in infants are costly to perform and ethically challenging. Inulin, fructo-oligosaccharides, and galacto-oligosaccharides have been the subject of several randomised controlled trials and are generally held to be the main substrates to have prebiotic potential during infancy. However, these agents are costly to produce and particularly in the case of FOS and GOS due to their small size, they confer a luminal osmotic effect and are rapidly fermented. These features carry the risk of precipitating undesirable clinical effects such as diarrhoea and abdominal discomfort. High amylose maize starch is not genetically engineered, can be readily cultivated, is slowly fermented and does not produce an osmotic effect. Furthermore, it can be readily incorporated into foods without altering the processing properties (Ashwar, Gani et al. 2016).

Prior to undertaking a clinical trial involing the feeding of HAMS to young infants, further evaluation of the results from this batch fermentation study could be obtained through the use of more complex, sophisticated simulators which more closely mimic human and, in particular, infant gut function. Due to their expense and technical requirements, these multistage continuous simulators are limited to a small number of laboratories.

The manipulation of the infant microbiota during a vulnerable period of development is not without risk. It is known from animal studies that dietary manipulation of the microbiota can affect brain development and neurobehaviour. Although, the exact mechanism(s) by which this occurs is not understood, one suggestion is that certain microorganisms produce metabolites which function as neurochemicals and can thereby affect brain function. In a recent study conducted in rodents, it was demonstrated that a diet supplemented with HAMS not only altered gut microbial composition, but also altered behaviour (Lyte, Chapel et al. 2016). In the study, six week old mice were administered a diet containing either normal corn starch or high amylose maize starch at a concentration consistent with other rodent studies which were designed to examine the nutritional properties of HAMS. The researchers demonstrated that in addition to taxonomic changes in the gut microbiota, the resistant starch diet was associated with an increase in anxiety like behaviours.

Unfortunately, other parameters of fermentation such as short chain fatty acid production and caecal pH were not measured. Interestingly, in children with autism spectrum disorder (ASD), a complex neuro-developmental disorder associated with a greater incidence of anxiety, it has been shown that total faecal short chain fatty acids are higher than in age matched controls (Wang, Christophersen et al. 2012). Although this does not prove a causal relationship between fermentation processes and cognitive function in humans, such cautionary results certainly merit consideration.

4.8 Conclusion

The composition of the complementary diet is a critical component of establishing the gut microbiota and is essential for examining how best to optimise it. This study has established that the faecal inocula of early weaning infants have the capacity to utilise HAMS and mHAMS as a potential substrate. While there was no significant difference in microbial composition between the test starches, both demonstrated the selective stimulation of *Bifidobacterium* and an increase in the Bacteroidetes:Firmicutes ratio; outcomes that if replicated in direct feeding studies, may be associated with beneficial health outcomes. The incubation of HAMS with pre-weaning faecal inocula did not significantly differ from the negative controls, suggesting that the capacity to ferment HAMS in this age group has not been confirmed.. Based on these results, we would recommend performing *in vivo* studies in infants to examine the short and long term effects of different doses of HAMS exposure during weaning on the composition and function of the emerging gut microbiota.

Chapter 5 The Relevance of the Colon to Zinc Nutrition

Gopalsamy, G. L., D. H. Alpers, et al. (2015). The relevance of the colon to zinc nutrition.Nutrients **7**(1): 572-583.

Abstract: Globally, zinc deficiency is widespread, despite decades of research highlighting its negative effects on health, and in particular upon child health in low-income countries. Apart from inadequate dietary intake of bioavailable Zn, other significant contributors to zinc deficiency include the excessive intestinal loss of endogenously secreted zinc and impairment in small intestinal absorptive function. Such changes are likely to occur in children suffering from environmental (or tropical) enteropathy (EE)—an almost universal condition among inhabitants of developing countries characterized by morphologic and functional changes in the small intestine. Changes to the proximal gut in environmental enteropathy will likely influence the nature and amount of zinc delivered into the large intestine. Consequently, I reviewed the current literature to determine if colonic absorption of endogenous or exogenous (dietary) Zn could contribute to overall zinc nutriture.

While I found evidence that significant Zn absorption occurs in the rodent colon, and is favoured when microbially-fermentable carbohydrates (specifically RS) are consumed, it was unclear whether this process occurs in humans and/or to what degree. Constraints in study design in the few available studies may well have masked a possible colonic contribution to Zn nutrition. Furthermore these few available human studies have failed to include the actual target population that would benefit, namely infants affected by EE where Zn delivery to the colon may be increased and who are also at risk of Zn deficiency.

Thus in conducting this review I have not been able to confirm a colonic contribution to zinc absorption in humans. However, given the observations in rodents and that feeding resistant starch to children is feasible, definitive studies utilising the dual stable isotope method in children with EE should be undertaken. However, preliminary to such an expensive research undertaking, it may be prudent to firstly investigate whether preceding zinc deficiency has the capacity to influence the colonic absorption of Zn within an animal model.

5.1 Introduction

As was mentioned in the introduction and literature review, zinc deficiency amongst young children is a major global public health issue. Given that fermentation of dietary fibre is associated with the increased absorption of divalent cations from the large intestine, I hypothesised whether such an approach, potentially using resistant starch, could increase zinc bioavailability. While the earlier chapters establish that young infants at the time of weaning have the capacity to ferment HAMS,

there has been no scientific review of the evidence as to whether such fermentation could result in increased absorption of zinc in humans.

It has been estimated that nearly one third of the world's population are receiving insufficient zinc from their diet (Brown, Rivera et al. 2004) . In many low income countries, intake of animal products, the major source of dietary zinc in Western countries, is very low. Further compounding this is the fact that bioavailability of zinc from vegetarian diets, predominantly based on cereals and legumes, is affected by phytic acid—an absorption inhibitor due to its avid affinity for divalent cations, including zinc (Hunt 2003). Due to their high demand for zinc and limitations in body stores, children are particularly vulnerable to the consequences of zinc deficiency. Restricted growth, immune dysfunction, diarrhoea, and an increase in mortality are all associated with clinical zinc deficiency in children (Fischer Walker, Ezzati et al. 2009). Given the relevance of zinc deficiency to global health, considerable effort has been devoted to better understanding zinc nutrition, including zinc homeostasis. While reduced bioavailability of dietary zinc is important, environmental enteropathy, a near universal condition among inhabitants of developing countries, might also affect zinc homeostasis due to changes in small intestinal morphology and function (Ramakrishna, Venkataraman et al. 2006; Ramakrishna, Subramanian et al. 2008).

One question to emerge from such conjecture is whether the large intestine could provide an auxiliary role for zinc absorption. Although evidence for colonic absorption of calcium, another divalent cation, exists in humans, there is little data to suggest a similar capacity for zinc absorption in the colon (Hylander, Ladefoged et al. 1990). However, as will be discussed, ignoring the colon's potential to participate in zinc homeostasis may be premature, particularly as several animal studies strongly suggest that colonic absorption of nutritionally significant amounts of zinc can occur (Hara, Konishi et al. 2000) (Yonekura and Suzuki 2005).

Thus the purpose of this review is to re-evaluate the current literature regarding the potential role of the human colon in zinc nutrition and consider what contribution, if any, the colon could make to improving zinc nutrition. It will also highlight particular areas of controversy and suggest areas where further research is required.

5.2 Intestinal Zinc Homeostasis

The gastrointestinal tract is crucial for the homeostatic control of zinc and involves a complex interplay of host (including disease states), dietary and other environmental factors (Krebs 2000). Apart from inadequate dietary intake of bioavailable zinc, other significant contributors to zinc deficiency may include the excessive intestinal loss of endogenously secreted zinc or impairment in small intestinal absorptive function (Manary, Abrams et al. 2010). Both of these conditions can occur in children suffering from environmental (or tropical) enteropathy, a condition characterized

by blunting of the villi, and T-cell infiltration of the small intestine, which afflicts many inhabitants of developing countries (Ramakrishna, Venkataraman et al. 2006). Maintaining adequate zinc for bodily functions is dependent on absorption of some proportion of exogenous (dietary) zinc and reabsorption of endogenous zinc secreted into the gastrointestinal lumen. The latter likely arises mainly from digestive enzymes and cell shedding (Krebs 2000). Ineffective homeostatic mechanisms aimed at conserving zinc or augmenting its absorption is evidenced by the widespread prevalence of zinc deficiency, particularly in vulnerable populations, such as children on marginal diets in countries where environmental enteropathy is prevalent (Lindenmayer, Stoltzfus et al. 2014)

If the colon is to participate in the absorption of luminal zinc, certain criteria must be satisfied: zinc delivered to the colon must be present in biologically important quantities, and the colonocyte must have the capacity to absorb this zinc and transfer it to the portal circulation. The zinc status of the host and by inference the body's demand for zinc, may be an additional factor influencing colonic zinc absorption due to its effect on zinc transporters (Jeong and Eide 2013).While it has been reported that the zinc status of the host does not affect zinc absorption, the studies which helped derive this conclusion did not manipulate conditions to favour a colonic contribution to zinc absorption (King 2010).

Before examining the potential for the colon to contribute to zinc absorption, the present state of knowledge of zinc absorption in the more proximal segments of the gut will be examined, since delivery of zinc into the colonic lumen is likely to be influenced by changes in absorption and secretion proximally.

5.3 Dietary (Exogenous) Zinc

It is generally agreed that the majority of dietary zinc is absorbed in the proximal small intestine, through a transcellular saturable carrier-mediated mechanism (Lee, Prasad et al. 1989).Using a triple lumen steady state perfusion technique in healthy volunteers, the entire small intestine has been demonstrated to absorb zinc (Lee, Prasad et al. 1989). Although the jejenum appears to have the highest rate of zinc absorption, the duodenum is first exposed to zinc during the postprandial period and is likely to also contribute to zinc absorption (Lee, Prasad et al. 1989). During the ingestion of a meal, it is unlikely that the concentration of zinc in the lumen reaches the level required to saturate the transporters involved in zinc uptake from the lumen (Lonnerdal 2000). Many compounds found in the diet may affect the bioavailability of exogenous zinc and phytic acid (PA) is thought to be the most important of these (Lopez, Leenhardt et al. 2002). When the molar ratio of dietary PA:Zn is increased, zinc absorption in the small intestine is progressively reduced (Lopez, Leenhardt et al. 2002). In such a situation it would be expected that a greater portion of PA-bound exogenous zinc would be delivered into the more distal gut lumen.

5.4 Zinc Transporters

Absorption involves influx into the enterocyte across the apical membrane and then across the basolateral membrane and into the portal circulation (Cousins 2010). Zinc absorption, at least from the small intestine, is predominantly carrier-mediated and thus saturable (Lee, Prasad et al. 1989).There are two major families of zinc transporters: ZIP and ZnT (Cousins, Liuzzi et al. 2006).The ZIP family of transporters regulates the influx of zinc into the cytosol, either from the lumen, serum or intracellular compartments. The ZnT family of transporters mobilize zinc in the opposite direction, *i.e.*, they facilitate zinc efflux from the cytosol to the extracellular environment or into intracellular organelles (Cousins, Liuzzi et al. 2006). Within each family, multiple subfamilies exist and their abundance varies between organs. See fig 5-1.

Fig 5-1 has been removed due to copyright restrictions

Figure 5-1 Localization and transport of zinc in a mammalian cell. Cellular localization and function of ZIP and ZNT zinc transporter family members. Arrows indicate the direction of zinc mobilization. ZIP1, 2 and 4 are induced in zinc deficient conditions, while ZNT-1 and 2 members are induced by zinc administration. (John, Laskow et al. 2010)

Both ZIP and ZnT transporters are expressed along the entire length of the gastrointestinal tract including the colon, raising the possibility of Zn absorption at more distal sites, *i.e.*, colon, provided that the luminal zinc is bioavailable and absorption is not suppressed by homeostatic control mechanisms (Cousins 2010). Uptake from the intestinal lumen into the enterocyte is at least in part mediated by ZIP4, a protein located on the apical membrane of enterocytes (Cousins, Liuzzi et al. 2006). In the mouse small intestine, experimental dietary zinc deficiency results in upregulation of ZIP4 mRNA and protein, accompanied by localisation of the ZIP4 protein to the enterocyte apical plasma membrane (Dufner-Beattie, Wang et al. 2003). This homeostatic regulation might serve to increase zinc uptake from the lumen during periods of inadequate zinc intake, although whether this will be sufficient to maintain zinc nutrition is uncertain (Pfaffl and Windisch 2003).

We do not have parallel information in humans with zinc deficiency but it has been shown that an increase in dietary zinc intake can down-regulate expression of zinc transporters, including ZIP4 mRNA expression in the human small intestine (Cragg, Phillips et al. 2005). The effects of reduced or increased dietary zinc on ZIP4 expression in the colon have not been studied in either experimental animals or humans. Mutations in ZIP4 are associated with acrodermatitis enteropathica (ADE), an autosomal recessive disorder characterised by dermatitis, alopecia and diarrhoea (Krebs 2013). While this loss of ZIP4 function reduces uptake of dietary zinc from the lumen, oral administration of pharmacological quantities of zinc can correct zinc deficiency in affected patients with ADE (Krebs 2013), suggesting that other mechanism(s) of zinc absorption exist in addition to that mediated by ZIP4.

5.5 Kinetics of Colonic Zinc Absorption

Although the transporters involved in zinc absorption, particularly ZIP4, are also expressed in the colon (Yang, Zhang et al. 2013), it is unresolved as to whether absorption of zinc from the mammalian colon is predominantly transporter mediated or whether passive diffusion may also occur (Gisbert-Gonzalez and Torres-Molina 1996; Condomina, Zornoza-Sabina et al. 2002). Although the tight junctions between the epithelial cells of the large intestinal epithelium are much tighter than those of the small intestine, this does not preclude the possibility of passive zinc absorption (Field 2003).

Using *in vitro* techniques which allow measurement of the unidirectional influx of zinc across the intestinal membrane of the rat, Condomina *et al.* demonstrated that zinc uptake from the colon increased linearly with the luminal concentration, suggesting that colonic zinc transport occurs via non-saturable diffusion (Condomina, Zornoza-Sabina et al. 2002). In the human small intestine, the rate of zinc absorption saturates above a luminal concentration of 1.8 mM (Lee, Prasad et al. 1989). Condomina *et al.* demonstrated that zinc transport from the colonic lumen of the rat increases linearly above this concentration, with non-saturable diffusion demonstrated at luminal concentrations as high as 11.08 mM (Condomina, Zornoza-Sabina et al. 2002). However, others have suggested that absorption of zinc from the rat colon exhibits combined kinetics, *i.e.*, both saturable and non-saturable components (Gisbert-Gonzalez and Torres-Molina 1996).

The kinetics of zinc absorption in the human large intestine has not been studied and it is difficult to reconcile the disparate findings in rodents. Differences in experimental techniques, diets, zinc status and strains of animals may account for some of the divergent observations. If the human colon were to be capable of non-saturable absorption of zinc, this might contribute significantly especially if zinc delivery to the colon is increased due to malabsorption or increased endogenous losses. More research is needed to prove or disprove such a theory.

5.6 Colonic Absorption of Zinc in Rodent Models

Although colonic zinc absorption has been demonstrated *in vitro*, using rodent large intestine, it is not known whether colonic zinc absorption is of physiological significance (*i.e.*, quantitatively relevant), especially in the human. Some investigators have concluded that the overall contribution of zinc absorption from the caecum and colon is small (Davies 1980; Sorensen, Andersen et al. 1998). For instance, Sorenson using an *in vivo* mouse model, quantified whole body and intestinal segment uptake of zinc using gamma counting of dietary 65Zn and concluded that there was little evidence to support colonic zinc absorption in zinc-replete animals fed a standard rodent diet (Sorensen, Andersen et al. 1998). However, using different experimental approaches, others have

reached an alternative conclusion suggesting that physiologically significant absorption of zinc can indeed occur from the rat colon (Lopez, Coudray et al. 1998; Hara, Konishi et al. 2000; Coudray, Feillet-Coudray et al. 2006). Hara *et al.* reduced proximal zinc absorption in rodents using omeprazole to increase luminal pH and found that serum zinc was lower in those rats which underwent caeco-colectomy, compared to sham-operated rats (Hara, Konishi et al. 2000). They concluded that the colon may compensate for impaired zinc absorption in more proximal regions.

5.7 Promotion of Zinc Absorption by Microbially-Fermentable Substrates

The effects of altering colonic luminal conditions on zinc absorption in animals, supports the colon's potential to manifest clinically significant zinc absorption (Lopez, Coudray et al. 1998; Yonekura and Suzuki 2005; Coudray, Feillet-Coudray et al. 2006). Fermentable substrates are dietary carbohydrates that have resisted digestion and absorption in the upper gastrointestinal tract and upon entering the microbially-rich lumen of the colon, become available for fermentation (Cummings 1981). Substrates such as dietary fibre, resistant starch, fructans and pentosans are fermented by resident colonic bacteria to produce short chain fatty acids (SCFAs), *i.e.*, acetate, propionate and butyrate (Cummings 1981). As an energy source for colonocytes, SCFAs increase colonocyte cellular differentiation and mucosal cell proliferation (Scheppach 1994). They can also increase caecal blood flow and increase cation solubility by lowering luminal pH (Scheppach 1994). It has already been demonstrated that feeding fermentable substrates to rats enhances calcium and magnesium retention from the colon (Younes, Demigne et al. 1996). Several studies in experimental animals demonstrate the beneficial effect of the colonic fermentation of indigestible substrates on the absorption of zinc (Yonekura and Suzuki 2005; Coudray, Feillet-Coudray et al. 2006). Several of the studies were conducted in animals fed diets containing a high PA: zinc molar ratio, limiting small intestinal absorption and thus ensuring a substantial delivery of zinc to the colon. Hayashi *et al.* also demonstrated that certain fibres could improve the growth rate in rats fed a high PA: zinc diet (Hayashi, Hara et al. 2001). Such findings do not clarify the mechanism or kinetics of zinc absorption from the colon, but provide evidence that interventions known to affect colonic luminal conditions can increase zinc retention.

5.8 Relevance of Zinc Status to Colonic Zinc Absorption

Although a reduction in dietary zinc has been shown to markedly increase fractional zinc absorption in humans (FZA), some experts assert that the zinc status of the host does not affect total zinc absorption (King 2010). Such studies, however, measured total intestinal zinc absorption, and have not been undertaken in a manner that exposes the host to prior factors that could increase the colon's contribution to zinc absorption, while perhaps minimising the small intestine's

role in such absorption. Combining such conditions with manipulation of zinc status may confirm whether colonic absorption of zinc is subject to regulation by zinc status. It cannot be assumed that mechanisms and regulation of colonic zinc absorption are identical to those in the small intestine. In mice the change in level of expression of zinc transporters in response to zinc deficiency was higher in the colon than in the small intestine (Pfaffl and Windisch 2003). It was also shown in mice that mRNA expression of metallothionein, an intracellular protein that may influence zinc absorption by buffering intracellular zinc, was down-regulated almost seven-fold in the colon but only 1.4-fold in the small intestine (Pfaffl and Windisch 2003). If however, there is significant passive absorption of zinc in the colon, independent of specific zinc transporters, then high concentrations of bioavailable zinc in the colonic lumen might facilitate absorption of zinc from the colon. Of course, in the absence of evidence to either support or refute such an observation, it remains only speculative.

5.9 Phytase-Mediated Phytate Hydrolysis

Carbohydrates and their fermentation products provide a rich energy source for resident bacteria in the colon, some of which produce phytase, an enzyme capable of hydrolysing PA to phosphate and inositol phosphates (Wise and Gilburt 1982). The human gastrointestinal tract has very limited endogenous phytase activity (Sandberg and Andlid 2002). The intermediate breakdown products of PA (inositol-6-phosphate), are more soluble and bind to minerals less avidly (Lonnerdal 2000). In a poultry model, it has been shown that feeding microbial phytases achieves luminal hydrolysis of phytate and facilitates increased retention of Ca, P, Mg and Zn by presumably increasing their bioavailability (Viveros, Brenes et al. 2002).

Bacteria are not the only source of luminal phytase as some plant foods also contain phytase (Kumar, Sinha et al. 2010). Unfortunately, while dietary phytase is active in the upper gastrointestinal tract, especially in the acidic gastric environment, they are inactivated in the more alkaline pH of the small intestine (Vohra and Satyanarayana 2003). Thus, microbial phytases might play a greater role, and it has been suggested that a potential mechanism by which fermentable substrates may improve zinc bioavailability and hence absorption is by promotion of phytase-mediated PA breakdown in the colon (Zacharias, Lantzsch et al. 1999).

5.10 Endogenous Zinc and the Colon

Under normal physiological circumstances, the primary site of absorption of exogenous (dietary) zinc in the human is thought to be in the proximal small bowel (Krebs 2000). Zinc is also delivered to the lumen from endogenous sources, primarily from pancreatic (zinc-enzymes and metallothionien) and biliary secretions, sloughed cells, mucus and mucosal zinc secretion (Taylor,

Bacon et al. 1991). Thus a portion of endogenous zinc may require absorption at sites more distal to dietary zinc. The chemical form of endogenous zinc will also differ from that of dietary zinc and some of this endogenous zinc may also be trapped within an insoluble organic matrix, such as within shed epithelial cells.

The amount of endogenous zinc in the lumen can be considerable (Krebs 2000). Certain disease conditions are likely to impact on the magnitude of endogenous zinc and the amount of such zinc entering into the colon. More than 10^{11} enterocytes may be shed into the lumen on a daily basis, a number that may greatly increase during periods of infection or in disease such as environmental enteropathy (Umar 2010). Given that each eukaryotic cell has at least 200 µmoles of zinc, the daily amount of endogenous zinc entering the gastrointestinal lumen via these shed cells alone may amount to several milligrams (Outten and O'Halloran 2001; Umar 2010). Bacteria present in the large intestine are capable of digesting sloughed epithelial cells The fate of the intracellular zinc released from such digestion is unknown. If intake of bioavailable dietary zinc is low or there is active small intestinal disease reducing proximal zinc absorption, conservation of this endogenous zinc by the colon could be of particular importance to maintenance of adequate zinc nutrition.

5.11 Zinc Absorption in Human Colon

There is a scarcity of human data to corroborate the findings in animals that the large intestine can participate in overall zinc absorption. The human colon certainly absorbs calcium (Hylander, Ladefoged et al. 1990) . Zinc absorption has been investigated in only a small number of subjects with extensive small bowel resection, and it is not possible to confidently deduce a role for the colon in zinc “salvage” from these results (Sandstrom, Davidsson et al. 1990). A single study found that absorption of zinc in patients following small bowel resection was similar to that in healthy subjects but as the purpose of the study was not to look at a colonic contribution to zinc absorption, the degree to which the colon remained in continuity varied and adaptation in the remaining small bowel might have been adequate to maintain overall zinc absorption (Sandstrom, Davidsson et al. 1990). Sandstrom, in a separate study instilled a radiolabelled zinc solution directly into the colon of healthy volunteers and measured whole body retention of the radionuclide. These investigators concluded that the human colon did not have the capacity to absorb clinically significant zinc (Sandstrom, Cederblad et al. 1986). More recently, it was found, by measuring retention of orally administered stable zinc isotopes, that there was no beneficial effect of a fructo-oligosaccharide on the absorption of zinc in postmenopausal women (Ducros, Arnaud et al. 2005). Similarly a study in young healthy male volunteers given inulin over a 28 day period found no beneficial effect on zinc absorption or balance (Coudray, Bellanger et al. 1997).

Although these human studies offer no support for biologically significant zinc absorption from the human colon, there are several pertinent points that must be made, apart from the fact that only a very limited number of subjects were studied. In Sandstrom's study the bowel preparation and hence clearing of luminal content including SCFAs might have affected the results (Sandstrom, Cederblad et al. 1986) .

The human studies undertaken so far did not explore the effect of zinc status of the participants and/or failed to create conditions that could maximise delivery of dietary zinc to the colon. The animal studies certainly suggest that colonic zinc absorption is likely to be of greater significance when small bowel absorption of zinc is somehow impaired (Hara, Konishi et al. 2000; Yonekura and Suzuki 2005). Major differences exist between rodents and humans in lifespan, body proportion, enteric composition and intestinal morphology. These differences may also be responsible for the conflicting observations between animal and human studies (Corpet and Pierre 2005). Systematic consideration of the multiple variables that might influence colonic zinc absorption and incorporation of these possible factors into the study design of future human studies will help resolve some of these issues.

5.12 Future Studies of Colonic Zinc Absorption

Several studies have investigated the importance of the colon in the absorption of other divalent cations, such as calcium. In a study of 118 patients with small bowel resections of various lengths, of which 38 had an ileostomy and 80 had part or whole of the colon remaining, Hylander *et al.* found that in patients with extensive small bowel resection the absorption of ^{47}Ca was significantly higher when the colon was preserved (Hylander, Ladefoged et al. 1990). There exists no study with similar methodology and with such a large number of participants, in the zinc literature. High doses of oral zinc have been demonstrated to overcome the absorptive defect in patients with acrodermatitis enteropathica (ADE), but there is no information whether the site of absorption of oral zinc in patients with ADE is the small intestine and/or the colon. Thus, will oral zinc correct zinc deficiency in a patient with ADE who has an ileostomy?

Colonic perfusion studies would be informative for zinc absorption if they could be done while maintaining the usual luminal environment (*i.e.* non-fasting and without bowel wash-out) but this might not be feasible especially in children. The effect of varying phytate intake would also be of interest. Certainly the dual isotope urine enrichment method has been extensively used to study zinc absorption in adult humans. Although, it does not distinguish between sites of absorption along the gut, it is still capable of capturing the colon's contribution to this absorption. Thus one could utilise this well-validated method to determine if factors that could promote increased bioavailability of zinc from the colon could improve overall zinc absorption.

5.12.1 Dual isotope enrichment method.

The absorption, distribution and elimination of minerals in living organisms can be studied using isotopically enriched tracers. A fundamental principle of all stable isotope techniques is to administer a stable isotope labelled compound to the body (orally or intravenously) in trace amounts to minimally disturb the physiology and subsequently track the fate of the compound or its products in urine, faeces or blood. In a dual isotope study the intravenous tracer is the isotope of lowest natural abundance, and the oral tracer is the isotope of second lowest abundance. The relative fraction of the oral compared with IV tracer dose in a 24 h urine pool is determined and represents the fraction of the oral tracer dose that was absorbed

Although, the dual isotope method does not distinguish between sites of absorption along the gut, it is still capable of capturing the colon's contribution to this absorption. Thus one could utilise this well-validated method to determine if factors that could promote increased bioavailability of zinc from the colon could improve overall zinc absorption. Such a study design could involve feeding children with EE, two diets with either high or low fermentative capacity, both with equal high PA:Zn ratios, and then measure zinc absorption using the dual isotope enrichment method once a steady state in fermentation was achieved. The reasoning for the high PA:Zn ratio would be to mimic the usual diet of these children and minimise absorption of zinc in the small intestine due to compromised bioavailability. It must be ensured that both groups receive equal amounts of zinc in the diet and that this amount of zinc is sufficient to increase caecal zinc pool size.

5.13 Luminal Conditions in the Large and Small Intestine

The luminal conditions of the large intestine differ markedly from the small intestine. The composition and thickness of the mucus layer within the large intestine is likely to be different to that found in the proximal gut and subsequent interaction of minerals with the epithelial cell wall is also likely to differ. The transit time in the colon is much longer than for the proximal gut, potentially facilitating a prolonged interaction of bioavailable minerals (such as zinc) with its apical membrane transport system. The presence of potential ligands such as SCFAs, the concentrations of coexisting cations and the activity of microbial derived phytases may also influence mineral absorption.

It is also possible that fermentative digestion of host proteins by the gut microbiota could liberate endogenous zinc from zinc-protein complexes since it is known that fermentation of dietary proteins occurs (Le Leu, Brown et al. 2007). But the degree to which bacteria resident in the gut may sequester this zinc for their use or make it available for absorption is not known (Monira, Nakamura et al. 2011). Alterations in the microbiota by the provision of a greater or lesser quantity of luminal zinc in the colon, and the clinical consequences have not been adequately studied.

Furthermore children with environmental enteropathy have a gut dysbiosis, and how this may affect the release of zinc from phytate or endogenous proteins is also unknown known (Monira, Nakamura et al. 2011). Ultimately the amount of zinc that enters the colon is dependent on the quantity of exogenous and endogenous zinc delivered into the lumen and the homeostatic control of their absorption and secretion proximal to the colon. Although the use of stable zinc isotopes can enable separate examination of net faecal excretion of endogenous and exogenous zinc, studies employing this technique were not designed to discriminate between the possible role of the colon in endogenous and exogenous zinc absorption, particularly in conditions that could facilitate colonic zinc absorption.

5.14 Conclusions

This review concludes that significant zinc absorption does occur in the rodent colon, particularly when fermentable substrates are fed. Whether it is influenced by whole body zinc status remains to be determined. In acknowledging the considerable limitations in the human studies to date, it remains unresolved as to whether feeding fermentable substrates could improve zinc bioavailability in those who are at risk of zinc deficiency, have impaired small intestinal function and/or experience substantial endogenous losses. These are the very conditions faced by many children in low-income countries.

The few available human studies which have led to general dismissal of the colon's capacity to participate in zinc absorption have involved healthy volunteers and not participants that might demonstrate a greater capacity to mobilize any latent ability of the colon to contribute to zinc absorption. Such a population could include zinc deficient infants affected by EE, a condition where zinc delivery to the colon may actually be increased. Thus further discriminative studies may still be required. While the powerful dual stable isotope method could be utilised in children with preceding zinc deficiency and EE, it may be prudent to use a less costly animal model to firstly determine if there is evidence that preceding zinc status could indeed modify colonic zinc absorption. Evidence of apparent absorption of other divalent cations, such as calcium and magnesium by the rat colon appears to correspond with eventual demonstrable absorption of these same minerals in the human large intestine (Younes, Demigne et al. 1996; Coudray, Demigne et al. 2003; Coxam 2007). The potential failure of zinc to follow such a pattern may itself be of some interest.

.

Chapter 6 The effect of preceding zinc deficiency on the capacity of a fermentable fibre to improve zinc status in a weaning rat model.

6.1 Abstract

Background/Aims: In humans, dietary fermentable substrates have been shown to increase colonic absorption of calcium and magnesium but not zinc. The absorption of zinc from the large intestine might depend upon the zinc status of the host. Thus the aim of this rodent study is to determine if any effect of resistant starch on zinc absorption is affected by preceding zinc status.

Methods:32 male Sprague Dawley rats were fed a zinc deficient or a zinc replete diet for 9 days (n=16/diet).Each group was then subdivided to receive either a diet containing a 10% resistant starch (RS) or control starch (CS) for 12 days.

Results: Compared to the CS group, the RS group increased femur zinc in rats with and without preceding zinc deficiency (p=.002).There was no interaction between preceding zinc status and the promotion of femur zinc by RS (p= .276). The effect of RS on caecal total SCFAs was significantly greater in animals with no preceding zinc deficiency, $p<0.05$.

Conclusion: In rodents colonic fermentation of RS does increase femur zinc, presumably through increased absorption of zinc from the colon. This increased absorption of zinc is not influenced by preceding zinc status. The parameters of fermentation were however influenced by preceding zinc status-suggesting that the capacity of microbes to ferment a dietary substrate is influenced by zinc availability.

6.2 Introduction

Zinc deficiency is a major health issue in low income countries, contributing to over half a million deaths per year in infants and children under five years of age (Krebs, Miller et al. 2014). Given this significance to global health, novel methods to improve the bioavailability of dietary zinc are still being sought. Certain dietary substrates that are fermented by colonic bacteria have been shown to promote the intestinal absorption of minerals, including zinc (Scholz-Ahrens, Schaafsma et al. 2001) (Seal and Mathers 1989). However, as opposed to other minerals such as magnesium and calcium, evidence for the facilitation of zinc absorption by such dietary fermentable substrates has so far been confined to laboratory animals (Hara, Konishi et al. 2000; Hayashi, Hara et al. 2001; Yonekura and Suzuki 2005). Recently, the hypothesis that resistant starch, a fermentable substrate, may increase net absorbed zinc in children at risk of zinc deficiency was tested (May, Westcott et al. 2015). In a small controlled clinical trial conducted in Malawi, significantly stunted children aged 3-5 years of age were given added dietary resistant starch over a period of 2 weeks (May, Westcott et al. 2015). The resistant starch was in the form of high amylose maize starch at a dose of 8.5 grams/day. The RS was added to a locally produced fried cake. Utilising dual zinc isotope methodology, the study found that the consumption of RS did not alter zinc homeostasis (May, Westcott et al. 2015). Notably, despite being stunted the children were not zinc deficient prior to involvement in the study.

To further understand this inconsistency between human and animal data, we set out to determine whether preceding intake of low dietary zinc could affect a colonic contribution to zinc absorption. In humans, it has been widely accepted that zinc is absorbed in a saturable (carrier) process from the proximal small intestine, and that current zinc intake is the primary determinant of zinc absorption (Krebs 2000; King 2010). Zinc homeostasis is instead maintained by adjusting the loss of endogenous zinc into the lumen (Hambidge, Miller et al. 2010). However, the small and large intestine of the gastrointestinal tract, have evolved to have discrete anatomical and functional differences, and as such, the findings from one area of the gastrointestinal tract may not be always extrapolated to the other. Under conditions where the absorption of zinc is shifted to more distal segments of the gastrointestinal tract, the effect of zinc status on its absorption has not been previously considered. It is also necessary to consider what the effect of preceding low intake of dietary zinc will have on microbial fermentation capacity. This has not been previously studied. Given the essential nature of zinc for the growth of most organisms, the availability of luminal zinc could have profound implications for the composition and function of the gut microbiota. For the purposes of this study, we elected to use femur zinc to detect a change in net zinc absorption over the course of the study period. It has previously been demonstrated that in addition to being a marker of zinc status, there is a good correlation between femur zinc and apparent zinc absorption (Yonekura and Suzuki 2005).

Accurate measurements of stool output, a requirement for mineral balance studies, are sometimes problematic in the setting of dietary fermentable substrate interventions due to increased faecal content and occasional diarrhoea (Yonekura and Suzuki 2005). Thus, the purpose of this rodent study is to determine whether (i) preceding zinc deficiency influences the capacity of resistant starch to influence zinc absorption, as measured by femur zinc; and (ii) preceding zinc deficiency can influence colonic microbial fermentation of resistant starch.

Specific Aim 1: To use weaning rat model to confirm whether dietary resistant starch can improve zinc status, as measured by femur zinc.

Hypothesis: In the setting of a high PA:Zinc diet, fermentation of RS will enhance the bioavailability of colonic zinc, increasing the colonic contribution to zinc absorption and improving zinc status.

Specific Aim2: To use a weaning rat model to determine whether preceding zinc deficiency alters the capacity of fermentation of RS to contribute to zinc bioavailability

Hypothesis: Previous studies suggest that the absorption of zinc from the rodent colon may be up regulated by preceding zinc deficiency. Thus preceding zinc deficiency may enhance any effect of a fermentable substrate on the colonic contribution to zinc absorption.

Specific Aim 2: To use weaning rat model to determine whether preceding zinc deficiency affects the capacity to ferment dietary high amylose maize starch.

Hypothesis: Zinc deficiency will affect the composition of the microbiota and may interfere with the fermentation of dietary substrates.

6.3 Materials and methods

6.3.1 Animals and Diets

The study was approved by the Flinders University Animal Ethics Committee (849-13). Male Sprague Dawley rats (4 weeks old), each weighing approximately 100 grams (University of Adelaide, Adelaide, Australia) were assigned to 4 groups (n=8). The rats were grouped to ensure that starting body weights were equivalent (see Appendix 8.9) and were housed in pairs in stainless-steel wire-bottom cages to limit coprophagy. A stainless-steel platform was constructed and placed in each cage to provide respite and shelter. The animals were housed in a 12 hour day-night cycle with controlled temperature and humidity, and were provided with free access to deionised water. A stainless-steel sipper with no rubber stopper was used to minimise zinc contamination.

In the first stage of the experiment, half of all the rats received a Zn replete diet (~Zn 50 ppm) while the other half received a Zn deplete (Zn 1.5 ppm) diet for a period of 8 days. The two diets differed only in their Zn content, (see Table 6-1), and were egg white-based to ensure low background levels of Zn. Egg white protein was purchased from The Melbourne Food Depot, Australia. Additional biotin (Sigma) was added to the diets to avoid a potential biotin deficiency resulting from the high avidin content in egg white protein. On day nine, a morning tail vein bleed was performed on eight randomly selected rats from both the Zn replete and the Zn deplete groups. Subsequently, the Zn deplete and Zn replete rats were divided into two further groups; one providing a 10% RS diet, and the other, a control diet containing corn starch and no RS (see Table 6-3). The fermentable starch selected for the study was Hylon VII, a high amylose maize starch which contains approximately 50% RS (Ingredion, USA). Adequate elemental Zn was added to both the CS and RS diets to ensure recovery from potential preceding zinc deficiency (see Table 6-3).

Table 6-1 Composition of diets for first stage of study.

	Diet Groups	
Component (g/kg diet)	**Zinc Deficient g/kg**	**Zinc replete g/kg**
egg white solids spray dried	200	200
dextrose monohydrate	625	625
Biotin	.004	.004
corn oil	100	100
vitamin mix[a]	10	10
mineral mix[b]	35	35
Cellulose	30	30
Zinc content (mg/kg diet)[c]	1.5±.1	49 ±.3
Phytate	0	0

[a.]Vitamin mix (mg/g):vitamin A palmitate, 0.8; vitamin D3, 0.25; Vitamin E, 5; Vitamin B1, 0.6;[a.]Vitamin mix (mg/g):vitamin A palmitate, 0.8; vitamin D3, 0.25; Vitamin E, 5; Vitamin B1, 0.6; Vitamin B2 0.6; Vitamin B6, 0.7; Vitamin B6, 0.7; Vitamin B12, 500 uL; nicotinic acid, 3; Calcium pantothenate, 1.6; Folic acid 0.2; Biotin 0.02; Vitamin K1, 0.075; Granulated sugar, 987. [b]See Table 6-2 [c] Values are means ± SEM, n=3

Table 6-2 Mineral mix composition for first stage diet (Zinc deficient and Zinc replete diets)

	Diet Groups	
Component (g/kg diet)	**Zinc Deficient (g/kg)**	**Zinc Replete (g/kg)**
Zinc sulfate heptahydrate	.25	5.7
Calcium HPO4.2H20	550	550
Sodium chloride	74	74
Potassium citrate monohydrate	220	220
Magnesium oxide	24	24
Manganese carbonate	3.5	3.5
Ferric citrate	6	6
Potassium sulphate	52	52
Cupric carbonate	0.004	0.004
Potassium iodate	0.01	0.01
Sodium selenate	0.01	0.01
Chromium potassium sulphate	0.55	0.55
Granulated sugar (sucrose)	69.7	64

Table 6-3 Dietary composition of diet for second stage of study. (resistant starch or control starch diet)

	Diet Groups	
Component (g/kg diet)	**Resistant Starch (10%)**	**Control Starch**
Corn Starch	300	500
Hylon VII - high amylose maize starch[a]	200	0
Corn oil	50	50
Casein	200	200
Phytic acid (dodecasodium salt from rice) = 90% phytate[b]	8	8
Mineral mix[c]	35	35
Vitamin mix[d]	10	10
Zinc sulphate heptahydrate	0.04	0.04
Cellulose	50	50
Choline	2	2
Ethoxyquin	0.01	0.01
Sucrose	143	143
PA:Zn	15	15
Zinc content (mg/kg)[c]	40.5± .2	40.9±.3

[a]Hylon VII approximately 50% resistant starch, 10% resistant starch diet. Ingredion,
[b]Phytic acid – Sigma. [c]Commercial mineral mix (Harlan, USA) (g/kg): calcium phosphate dibasic, 500; sodium chloride, 74; potassium citrate monohydrate 220; potassium sulfate, 52; magnesium oxide, 24; manganese carbonate3.5; ferric citrate, 6; zinc carbonate, 1.6; cupric carbonate, .3; potassium iodate, 0.01; sodium selenite, 0.01; chromium potassium sulfate 0.55; sucrose, 118. [d]Vitamin mixture: same as in Table 6-1 [c] Values are means ± SEM, n=3

This second stage of the experiment continued for 12 days**.** After a 2 day adjustment period to the new diet, the rats were placed in individual cages and their daily intake was measured over a four day period. Food spilled through the wire bottom cages was collected and weighed. Food consumption was calculated as the amount of food spilled subtracted from the amount of food missing from the feeder. The rats were weighed on a weekly basis.

6.3.2 Specimen and tissue collection

Following 12 days of either the resistant starch or control starch diet and after an overnight fast, all the animals were euthanized by CO_2 asphyxiation. Blood was collected through a cardiac puncture. After a laparotomy the large intestine was resected and the caecum was excised and weighed. To calculate caecal digesta weight, the caecum was measured before and after removal of caecal digesta. The length of the colon was measured. A portion of collected caecal digesta was diluted in 3 volumes of internal standard solution (heptanoic acid, 1.68 mM, pH 7.0) and stored at -20^{0}C for later analysis of SCFA concentrations. The right femur was removed, cleaned, weighed and then freeze-dried.

6.3.2.1 Femur Zinc analysis

The right femur was freeze dried until steady weight was obtained. The dry bone weight was measured and it was then ashed at 550^0C in an acid-washed ceramic pot, and placed in a muffle furnace for 24 hours to determine the ash weight. A portion of ashed sample (10mg) was then dissolved in 1mL of high purity nitric acid for atomisation on a heating block at 60^0C for 2 hours. The amount of Zn in the femur sample solution was quantified with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Perkin Elmer Elan DRC).

6.3.2.2 Plasma Zinc and Dietary Zinc

Blood samples were obtained from a tail vein bleed on day 8 and a cardiac puncture at the end of the experiment. A trace metal free needle was used and blood was collected in a heparin containing tube. The samples were then centrifuged at 10,000 x g for 15 min to obtain plasma. The plasma was then digested in 1 mL of high purity nitric acid at 60^0C for 2 hours and then analysed by ICP-MS (Perkin Elmer Elan DRC instrument). Diets were freeze dried and subjected to the same digestion procedure and analysis as for the femur zinc. A commercial bovine liver powder quality control with known Zn concentrations was analysed with every run. Wet digestion and analysis of zinc using ICP-MS was performed at SA Pathology, Adelaide, Australia.

6.3.2.3 SCFA analysis and calculation

Caecal digesta samples containing 3 x volume for weight of 1.68 mM heptanoic acid were mixed and centrifuged at 3,000 rpm for 10 minutes at 5^oC. 300 μL of the supernatant with (10μL of 1M phosporic acid) was then placed into filter tubes for further clean-up. Samples were subsequently loaded onto the Agilent 6890N Network gas chromatograph system with automatic loader/injector. The GC column was a Zebron ZB-FFAP (Phenomenex, Lane Cove, NSW, Australia), length 30 m, internal diameter 0.53 mm, film thickness 1 μmeter. The GC was programmed to achieve following run parameters: initial temperature 90^0C, hold 0.5 min, ramp 20^0C/min final temperature 190^0C, total run time 8.0 min. Gas flow 7.7 mL/min splitless to maintain 3.26 psi column head pressure, septum purge 2.0 mL/min. Calibration standards were prepared to give a mixture of the following concentrations of acids (mM): 26.22 acetic, 19.86 propionic, 3.24 isobutryic, 16.32 butyric, 5.40 isovaleric, 5.6 valeric, 4.74 caproic and 5.04 heptanoic. This standard mix (0.2 μlitres) was used to calculate retention times and to create a standard plot. A standard curve was used to determine the concentration of acids in the sample.

6.3.2.4 Statistical Analysis

The data are presented as the arithmetic mean and SEM for each treatment group. A value of $P < 0.05$ was taken as the criterion of significance. Two-way ANOVA was used to determine the effect of preceding Zn deficiency and the effect of dietary resistant starch on parameters of fermentation and Zn retention.

Significant interactions between RS and preceding zinc status were analysed using Scheffe's method for pair-wise comparisons to determine simple main effects. Analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago Il USA).

6.4 Results

6.4.1 Weight gain and food intake

Prior to the second dietary intervention, the weight of the rats in the zinc deficient group was significantly less than the rats which had received the zinc replete diet (120.4 vs 165.4 g, p<.001). Following the second dietary intervention, rats with a preceding zinc deficiency had lower food intake (g) (16.4 ±.21 vs 21.19 ±.32, p <.001) and less percentage weight gain (82.19 ±2.3 vs 152.2 ± 3.1 p<.001) than the zinc replete group, but resistant starch had no statistically significant effect on these parameters.

Table 6-4 Mean weight and food intake

	Preceding Zn Deficiency		Preceding Zn replete		Two Way ANOVA(P-values)[1]		
	Resistant Starch n=8	Control Starch n=8	Resistant Starch n=8	Control Starch n=8	Preceding Zn status	Diet	Zn status x Diet
Weight gain[2]	80.92± .59	83.45 ± 1.42	152.73 ± .62	151.67 ± .85	P <.001	P=.690	P=.533
Food intake (g/day)[3]	16.3 ± .34	16.7 ± .24	21.1 ± .58	20.9 ± .6	P<.001	P=.831	P=.248

[1]Means ± SEM, n=8. Preceding Zn Status: effect of preceding zinc deplete or Zn replete diet, Diet: effect of receiving either resistant starch or control diet, Zn status x Diet: effect of interaction, NS: not significant (p>0.05)
[2]Mean body weight gain at completion of study compared to start of study in the four groups (±SEM); n = 8 in each group
[3]Mean Food intake – measured over a four day period, following a two day adaptation to the experimental diet containing either control or resistant starch. (±SEM); n=8

6.4.2 Plasma zinc prior to second dietary intervention.

Prior to commencement of the second dietary intervention, plasma zinc from eight randomly selected rats from each the zinc deficient and zinc replete groups, confirmed that rats fed that were fed the zinc deficient diet were severely zinc depleted (6.6 vs 24.8 μmoles/L, p<.001).

6.4.3 Femur zinc, plasma zinc and fermentation

It was hypothesised that a fermentable substrate such as RS, might contribute to increased absorption of zinc, but that this effect might differ across rats with and without a preceding zinc deficiency. In relation to femur zinc, there was a significant main effect for RS when compared to highly digestible corn starch and a significant main effect for preceding zinc status, but the interaction between RS intake and preceding zinc status was not significant (see Table 6-6), i.e, preceding zinc status did not influence the potential for a RS intervention to increase femur zinc.

There was no significant correlation between plasma zinc and femur zinc (r=-.104, p=.577), (see Fig 6-1). This finding is consistent with the published literature, with plasma zinc only being an accurate marker of zinc status when severe zinc deficiency is present (Lowe, Fekete et al. 2009). In relation to plasma zinc, there was no main effect of either the resistant starch intervention or preceeding zinc status (see also Table 6-5).

Table 6-5 The effect of resistant starch and preceding zinc deficiency on femur zinc, femur ash weight and plasma zinc.

	Preceding Zn Deficiency		Preceding Zn replete		Two Way ANOVA (P-values)[1]		
	Resistant Starch	Control Starch	Resistant Starch	Control Starch	Preceding Zn status	Diet	Zn status x Diet
Femur Zinc per dry weight (ug/g DW)	148.70±7.7	124.33±5.0	161.28±3.78	148.94±4.22	P=.002	P=.002	P=.276
Femur Ash weight mg	141.25±3.0	148.75±3.5	195.0±2.67	193.75±2.63	P<.001	P=.30	P=.151
Plasma zinc (umoles/L)	23.0±1.9	24.3±0.68	21.7±1.17	22.0±1.9	P=.201	P=.450	P=.701

[1]Values are means±SE (n=8); Preceding Zn Status: effect of preceding zinc deplete or Zn replete diet, Diet: effect of receiving either resistant starch or control diet, Zn status x Diet: effect of interaction, NS: not significant (p>0.05);

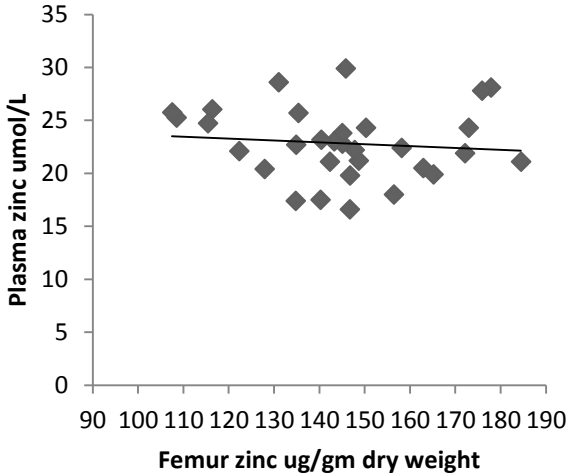


Figure 6-1 Relationship between plasma zinc and femur zinc.

There were no significant interactions between preceding zinc status and diet for faecal output, colonic length, caecal weight and caecal pH. However, except for caecal pH, for each of these variables there was a significant main effect for both zinc status and diet. Caecal pH was not affected by preceding Zn status, but was lowered by the RS intervention (see Table 6-6).

A statistically significant ordinal interaction was observed between preceding zinc status and diet for caecal digesta weight and total SCFAs (see Fig 6-2). This suggests that the extent of RS fermentation might be dependent on the preceding zinc status of the animal.

Table 6-6 Effect of preceding zinc status and resistant starch on markers of fermentation

	Preceding Zn Deficiency		Preceding Zn replete		Two Way ANOVA (P-values)[1]		
	Resistant Starch	Control Starch	Resistant Starch	Control Starch	Preceding Zn status	Diet	Zn status x Diet
faeces (g/day)	1.45 ±.18	.90 ±.07	2.48 ±.12	1.66 ±.15	P<.001	P<.001	P=.329
colon length (cm)	21.08 ±.27	19.10 ± .4	23.25 ±.63	22.63 ±.39	P<.001	P<.001	P=.747
caecal weight(g)	.96 ±.11	.39 ±.01	1.13 ±.05	.74 ±.04	P<.001	P<.001	P=.149
caecal pH	5.76±.21	6.69±.06	5.64±.09	6.34±.24	P=.177	P<.001	P=.497
Total SCFA (umol)	85.34 ±11.1	42.96±5.57	298.7±5.9	86.99±6.87	P<.005	P<.005	**P<.005**
caecal digesta weight	2.33 ± .29	.82 ± .11	6.47 ± .30	1.69 ± .11	P<.001	P<.001	**P<.001**

[1]Values are means±SEM (n=8); Preceding Zn Status: effect of preceding zinc deplete or Zn replete diet, Diet: effect of receiving either resistant starch or control diet, Zn status x Diet: effect of interaction, NS: not significant (p>0.05);

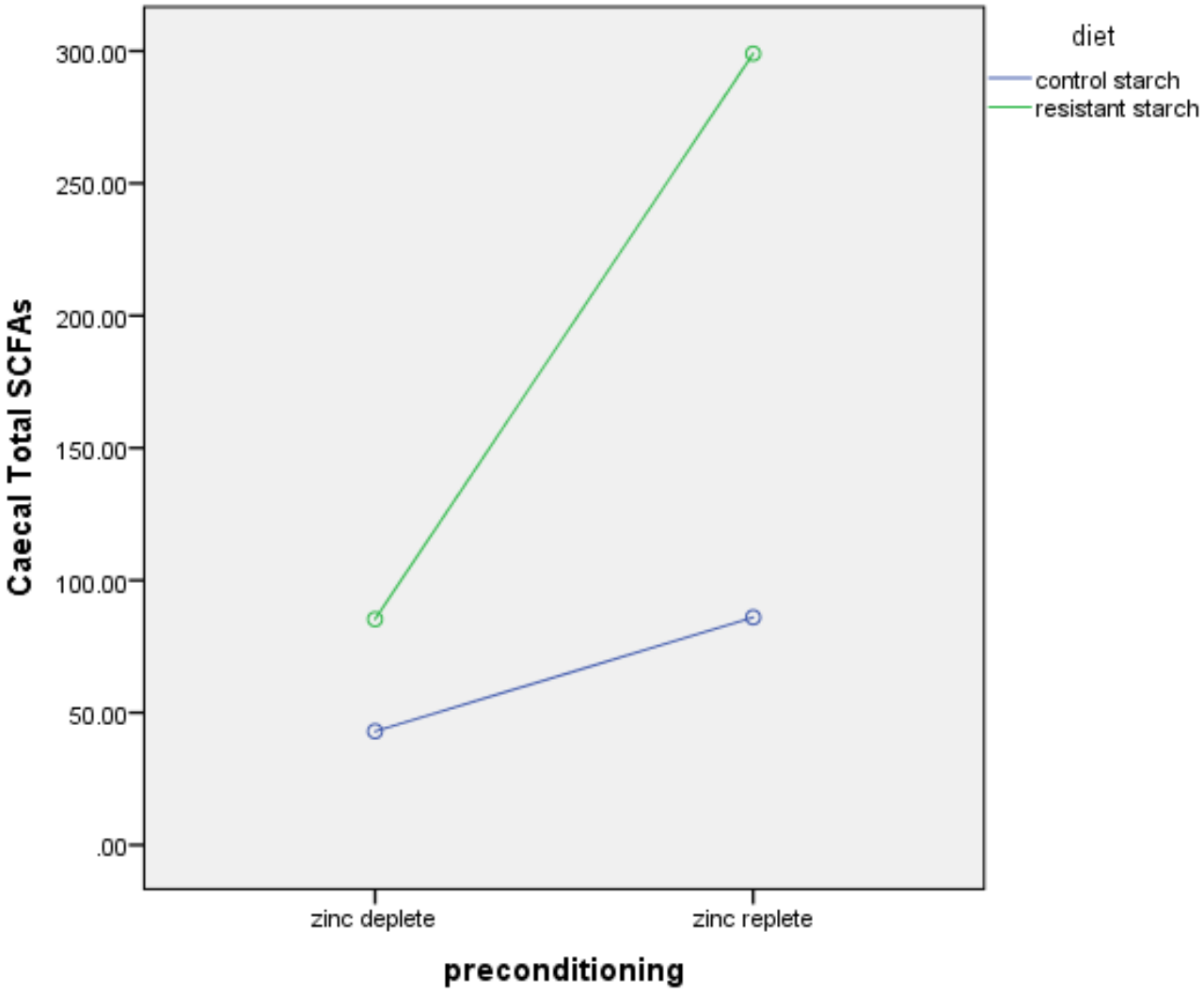


Figure 6-2 Graphical representation of the interaction between diet and preceeding zinc status in relation to Caecal Total SCFAs (mmol) – generated with SPSS

Post-hoc analyses using Scheffe's test determined a statistically significant increase in total SCFA content when the zinc replete/RS group was compared to the other three groups ($p<0.001$). The only statistically insignificant pairwise comparison was between the preceding zinc deficient/ RS group and the zinc replete/CS group ($p=1.0$). Scheffe's post-hoc analyses also revealed a statistically significant increase in caecal digesta weight between the preceding zinc replete/RS group and the other three groups ($p=<0.001$). There was also a significant difference in caecal digesta weight between the preceding zinc deficient/RS group and the preceding zinc deficient/CS group. This interaction between diet and preceding zinc status on total caecal SCFAs suggests that the fermentation of resistant starch might be dependent on the preceding zinc status of the animal.

6.5 Discussion

This experimental study conducted with zinc deficient and zinc replete rats during their growth phase confirms previous observations that the intake of resistant starch, a fermentable substrate, increases bone zinc content in rodents (Yonekura and Suzuki 2005). It has previously been demonstrated that bone zinc is a marker of zinc status and this correlates with apparent absorption of zinc during an experimental period (Yonekura and Suzuki 2005). Presumably, the resistant starch, through its fermentation by colonic microbes, improved zinc bioavailability by increasing the colonic absorption of zinc. Where this study is novel is in its further consideration of whether preceding zinc status can influence both fermentation of the resistant starch and absorption of zinc from the colon.

It has been claimed that zinc status does not influence zinc absorption, but rather that it is the quantity and bioavailability of dietary zinc that is the major regulator of absorption (King 2010). This is in contrast to iron, where iron deficiency can enhance iron absorption (Theil 2011). This finding that the absorption of zinc is resistant to the effects of zinc status is however based on studies which have not considered the possibility of colonic participation in zinc absorption. Phytate is a negative nutrient which chelates with dietary divalent cations, such as Zinc, to form an insoluble complex. Consequently, a high ratio of phytate to zinc in the diet increases the colonic pool of zinc by inhibiting its more proximal absorption. Administering such a diet (high Pa:Zn ratio), along with a fermentable substrate to both zinc deficient and zinc replete rats, creates the necessary conditions to determine whether when zinc absorption is shifted more distally in the GI tract, preceding zinc status may yet have an influence upon its absorption.

The few studies that have compared changes in the expression of zinc transporters between the distal and more proximal segments of the gastrointestinal tract have indeed found regional differences in the regulation of Zn transporters (Pfaffl and Windisch 2003). For example, in mice, the expression of zinc transporters following dietary zinc restriction, was higher in the colon than in the small intestine (Pfaffl and Windisch 2003). In the same study, it was also found that mRNA expression of metallothionein, an intracellular protein that may influence zinc absorption by buffering intracellular zinc in the face of zinc deficiency (Maret and Krezel 2007), was down-regulated several fold in the colon, but only 1.4 fold in the small intestine. More recently in pigs, animals whose digestive system is closer to humans than rodents, it was demonstrated that there is a 2.7 fold up-regulation of Zip4 in the colon during intake of low dietary zinc in comparison to only a 1.5 fold increase in the small intestine(Brugger, Hanauer et al. 2015).

Zip 4 is the primary zinc transporter involved in the uptake of zinc from the gastrointestinal lumen into the enterocyte, and, as such, has a key role in maintaining systemic zinc homeostasis in humans (Andrews 2008). It has previously been shown that the expression of ZIP4 mRNA and protein is down-regulated at both a transcriptional and a post-translational level when zinc is more available (Dufner-Beattie, Wang et al. 2003). Whether colonic expression of Zip4 is subject to similar rapid down-regulation has not been investigated.

The actual facilitation of zinc absorption by the fermentation of non digestible carbohydrates in rodents may occur through multiple means. As was demonstrated in this study, the predominant by products of carbohydrate fermentation, short chain fatty acids, are volatile organic acids that can alter the intestinal milieu to significantly reduce the pH of the intestinal contents (Scholz-Ahrens, Schaafsma et al. 2001). This acidification increases mineral solubility and can promote mineral absorption. SCFAs can also regulate cell proliferation, migration and survival as evidenced in our study by the increased length of the colon and size of the caecum, with the resistant starch interventions (Cook and Sellin 1998). In addition to increasing the total absorptive area available for absorption, these trophic effects of SCFAs may increase the expression of transporters involved in Zn uptake, such as Zip4. By also acting as a substrate for many species of colonic microbiota, the provision of dietary fermentable substrates such as RS can also significantly raise the concentration of bacteria in the colonic digesta. Unlike humans, various species of gut bacteria express microbial phytase, an enzyme capable of breaking down phytate to its lower moieties (Kumar, Sinha et al. 2010). These lower inositol groups bind minerals such as zinc less avidly. Once the zinc is released from an insoluble complex it may then be available for absorption. It is also known that SCFAs can form a soluble complex with another divalent cation, iron, to facilitate its entry into intestinal cells independent of a dedicated transporter (Bougle, Vaghefi-Vaezzadeh et al. 2002). Whether such a mechanism of absorption exists for zinc is not known.

However, despite hints for there being differences in the regulation of zinc transport in the colon when compared to the small intestine, the results of this study suggest that the preceding zinc status of the host does not influence apparent colon mediated absorption of zinc. This absence of an interaction between preceding zinc status and the promotion of femur zinc by a fermentable substrate has not been demonstrated previously. Such a finding aligns with the homeostatic mechanism that are thought to govern zinc absorption in the small intestine (Hambidge, Miller et al. 2010).

The data also demonstrated that total SCFA and digesta weight were significantly increased in the zinc replete rats following the RS diet. If the absorption of zinc from the colon was primarily transporter mediated and thus saturable, then greatly increasing caecal SCFA content may not result in further increases in bone zinc. However, it might also be that femur zinc as an endpoint lacks the resolution to detect more subtle differences in zinc absorption.

It might also indicate that preceding nutrient availability can affect the capacity of microbes to ferment dietary substrates. For the purposes of this study, zinc deficiency was achieved through the restriction of dietary zinc intake. However, in infants and young children, clinically significant zinc deficiency is more commonly a result of reduced zinc bioavailability, often due to an increased phytate:Zn ratio (Miller, Hambidge et al. 2015). Although the exact mechanism of zinc deficiency may not be immediately relevant to the host, it is likely to be of great significance to gut microbial composition and function. As mentioned earlier, members of the gut microbiota possess microbial phytase activity and thus, may be able to access zinc even if chelated to phytate (Kumar, Sinha et al. 2010). The expression and activity of microbial phytase can vary significantly amongst members of the microbiota (Vohra and Satyanarayana 2003). Resistance against incoming pathogens depends upon a stable endogenous microbiota. In low income countries, differences in dietary PA:Zn may have relevance beyond fermentation capacity and might influence the balance between protective microbiota and potential pathogens. Examination of changes in the composition and functional capacity of the gut microbiota in response to a low zinc diet versus a diet with a high phytate to zinc ratio was not a component of this study. This might be worth examining in future. In chickens, chronic zinc deficiency has a significant effect on caecal microbiota, leading to a greater abundance of Proteobacter, a significantly lower abundance of Firmicutes and a significant reduction in alpha diversity (Reed, Neuman et al. 2015). Such effects, if replicated in rodents, might have led to the compromised capacity to ferment the RS.

This study has several limitations. It did not include a paired rat model, where the daily intake of rats on the zinc deficient diet was measured and provided in equal amounts to rats fed a zinc replete diet. The inclusion of such an additional group may have clarified how much of the effect on fermentation capacity was due to zinc deficiency alone and how much was due to general malnutrition. However, in severe zinc deficiency, anorexia is a known side effect and as such the current study design reflects a real nutritional situation. Examining the microbiota for compositional changes might have also provided valuable insight into which bacteria involved in the fermentation of dietary substrates are more vulnerable to preceding zinc deficiency. Finally, whilst the use of wire bottom cages limits coprophagy, it does not completely prevent the practice. Consequently, it is possible that the greater absorption of zinc in the resistant starch group might reflect increased proximal absorption.

6.6 Conclusion

Interspecies differences limit the immediate translation of these findings to humans. However, the results are still informative. It appears that RS facilitated absorption of zinc from the mammalian colon is not responsive to zinc status. While this does not explain the discrepancy between animal and human studies in regards to colonic absorption of zinc, it provides some justification for not

pursuing costly clinical studies where human participants with preceding zinc deficiency could be recruited with the thought that this may reveal a latent capacity for colonic zinc absorption. However, there could be value in understanding how the availability of dietary zinc can affect microbiota dependent metabolic functions, as was demonstrated in this study. Finally, the availability of zinc and phytate may not only be of relevance to commensal organisms, but also for incoming pathogens. In low income countries where the burden of enteric disease is very high such research may be of major significance.

Chapter 7 Discussion

The transition from breast-feeding to solid food, through the introduction of complementary feeding, is a critical period in the assembly of the human gut microbiota (Subramanian, Blanton et al. 2015). Given the relationship between the early gut microbiota and the pathogenesis of several metabolic, autoimmune, and allergic diseases, it is imperative that the composition and timing of the complementary diet on future disease risk is considered (Ismail, Licciardi et al. 2013; El Aidy, Dinan et al. 2015; Ruff and Kriegel 2015). Nationally and internationally, there exist no guidelines, independent of the recommendation to exclusively breast feed until 4-6 months of age (Kramer and Kakuma 2012), regarding what constitutes the 'ideal' complementary diet (Symon and Bammann 2012).

It has been estimated that for much of human history, adult humans consumed more than 100 grams of dietary fibre on a daily basis (Eaton 2006; Whitton, Nicholson et al. 2011). This initially consisted of predominantly indigestible plant materials such as grasses, sedges and tubers, but eventually expanded to also include coarsely ground grains and legumes (Eaton 2006). In comparison, the modern Western diet contains no more than approximately 15-27 g of fibre per day (Whitton, Nicholson et al. 2011). An increasing number of publications, using observational data and dietary interventions, suggest that the early intake of fermentable fibres may have a beneficial effect on both the gut microbiota and the future health of the host (De Filippo, Cavalieri et al. 2010; Nauta, Ben Amor et al. 2013; Hallam, Barile et al. 2014). Despite such evidence, it is unlikely that as a population, adults in Western societies will consider returning to a diet of coarsely ground grains and legumes. To expect such population level change to early weaning practices may be equally unlikely. Prebiotic mediated manipulation of the emerging gut microbiota, with a fermentable fibre that might be readily incorporated into complementary foods could be an alternative option.

7.1 The prebiotic potential of HAMS in infants

As discussed in Chapter 2, prebiotics are non-digestible carbohydrates that stimulate the growth of beneficial bacteria in the large intestine (Gibson and Roberfroid 1995). They are capable of influencing maturation of the immune system, regulating allergic inflammation, and up-regulating the response of T regulatory cells (Hardy, Harris et al. 2013). High amylose maize, a type 2 resistant starch, is a fermentable fibre that has prebiotic potential (Zaman and Sarbini 2015). It has already been successfully incorporated into adult cereal products and breads (Yue and Warning 1998; Brown, Macnamara et al. 2000). This experience provides valuable information regarding manufacturing costs and how best to navigate issues of palatability and texture were such an intervention to also be used in infant food products.

It is during infancy that the intake of prebiotics might have the greatest impact on long term composition of the gut microbiota and the greatest potential to influence host immune function. Inulin, fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS), which are all bifidogenic, have been the most extensively examined prebiotics during infancy (Bertelsen, Jensen et al. 2016). As was discussed in the literature review, traditional prebiotics such as FOS and GOS have several limitations including their high cost of production, the rapidity of their fermentation, and their high osmotic effect by virtue of their small molecular size (Figueroa-Gonzalez, Quijano et al. 2011). High amylose maize starch offers several advantages over conventional infant prebiotics; it is not osmotically active, it is less rapidly fermented, and it can be produced for less cost on an industrial scale. In addition to increasing the production of beneficial metabolites such as short chain fatty acids, HAMS also has favourable effects on the microbiota, including in some studies, a bifidogenic effect (Bird, Conlon et al. 2010). Despite these findings, the potential for HAMS to function as a prebiotic during infancy, particularly during the weaning period, had not been previously considered. This thesis provided the framework to explore such a proposal.

7.1.1 *In vitro* examination of the capacity of infant faecal inocula to ferment HAMS and mHAMS

The capacity of HAMS and mHAMS to be fermented by infant faecal inocula was examined in Chapter 3. Using *in vitro* fermentation methodology, I demonstrated that the faecal inocula of pre-weaning and early to mid-weaning healthy infants have some capacity to ferment HAMS and acetylated HAMS, as evidenced by an increase in SCFA production following incubation with these substrates. T**his indicates that some of the beneficial health effects of resistant starch, and in particular HAMS, may be available earlier in the human lifespan than previously recognised.** Moreover, I established that the capacity to ferment HAMS is significantly enhanced with weaning, even when measured within 12 weeks of an infant commencing solids. Further analysis determined that within the pre-weaning group, there was no difference in the fermentation of HAMS between exclusively breast-fed infants and mixed-fed infants. However, there is a possibility of a type 2 statistical error as the study was not powered to address this question.

The major SCFA produced during the fermentation of the resistant starches both in the pre-weaning and weaning period by faecal inocula was acetate. There is evidence to suggest that acetate may have unique effects on the immune system, including the dampening of a pro-inflammatory response by intestinal epithelial cells (Arpaia 2014). The proportional contribution of butyrate to total SCFA concentration was very low, and much less than what has been previously reported during *in vitro* fermentation of starch by adult faecal inocula (Brouns, Kettlitz et al. 2002). This likely reflects the importance of microbial composition on the profile of SCFA production. In comparison to adult faecal inocula, both pre and weaning infants have a high prevalence of members of the Actinobacter phyla, which include acetate producers such as bifidobacteria.

Most butyrate producers in the human colon belong to the Firmicutes phylum and in particular clostridial clusters IV and XIVa. Clostridial clusters IV and XIVa are highly oxygen sensitive and strictly anaerobic. Consequently, these organisms are vulnerable to the in vitro environment where small amounts of oxygen may be present. The Firmicutes phylum is not well represented within the infant age group (Macfarlane and Macfarlane 2003). In adults, Faecalibacterium prausnitzii and Eubacterium rectal, together account for approximately 27% of the total faecal gut microbiota and are major contributors to butyrate production. In infants however, these bacteria and other important butyrate producers such as Roseburia spp, are much less abundant. Indeed in the case of F prausnitzii it has been shown that faecal numbers in infants younger than 6 months are undetectable and only slightly increase between the age of 6 and 24 months. (Rivière, Selak et al. 2016)

The relative contribution of acetate to the total pool of SCFA decreased post the commencement of solids, even in fermentations containing no additional substrate. This was due to the major change in composition and functional capacity of the weaning faecal microbiota and the occurrence of more cross-feeding whereby the metabolic products of fermentation by one species provide substrates to facilitate the growth of other populations (Gibson, Probert et al. 2004).

I also demonstrated that the faecal inocula of weaning infants have the capacity to ferment a chemically modified starch that is commercially available and widely used in the food industry. Such evidence may quell some of the concerns expressed by sectors of the community regarding the presence of modified starches in infant food products.

7.1.2 *In vitro* examination of the effects of HAMS and mHAMS on infant faecal microbiota

Chapter 4 examined the changes in the microbial community following incubation with HAMS or mHAMS for 24 hours. In the weaning infants there was a significant increase in bifidobacteria copy number following incubation with HAMS and mHAMS when compared to the negative control incubations. **This satisfies one of the essential criteria for a substrate to be classified as a prebiotic - a selective increase in the growth of bacteria associated with a health benefit (Gibson and Roberfroid 1995)**. Further clinical studies will be needed to confirm whether this translates into any health benefit.

NGS was utilised to further elucidate the changes to the microbiota following incubation of infant faecal inocula with the resistant starches. **Using pre-weaning faecal inocula, I found no major taxonomic change at 24 hours of incubation with HAMS compared to the negative controls.** This could be due to the known significant inter-individual variation in pre-weaning infant inocula masking substrate level changes or it could be that the substrate was unavailable for fermentation

due to the ‘functional immaturity’ of the microbiota. However, if the latter were the case, I would not have expected the pre-weaning infant inocula to produce more SCFA following incubation with the HAMS when compared to the negative controls. To resolve this question, a follow on study could measure the percentage of RS remaining in the fermentation tubes following incubation. This will confirm the degree to which the RS was consumed.

Chapter 4 revealed that significant differences in taxonomic composition following incubation of weaning faecal inocula with the resistant starches when compared to negative controls. Following incubation with both HAMS and mHAMS, at the phylum level, there was an increase in the relative abundance of Actinobacteria and Bacteroides and a reduction in Proteobacteria.

The Bacteroidetes:Firmicutes ratio was also increased following incubation with both of the starches. At the genus level, there was a significant increase in the abundance of Bifidobacterium and Bacteroides. If these changes are replicated during direct infant feeding studies, there may be potential measurable health benefits. As was mentioned, **it does not appear that the prebiotic effect of HAMS extends to the faecal inocula of pre-weaning infants**. Currently, commercial infant formula containing exogenous oligosaccharides are administered to infants who have not yet commenced solids. Based on these *in vitro* findings, such a strategy would not be effective if HAMS was to be used as the substrate.

7.1.3 Future research priorities

Experimental animal studies demonstrate that the provision of a particular fibre during weaning may increase the capacity for caecal fermentation of that fibre in adulthood, even if dietary intake of the fibre is normalized during the interim period (Armstrong, Eastwood et al. 1992). There is significant inter-individual variation in the capacity of adults to ferment resistant starch and this may reflect differences in the intake of resistant starch during the establishment of the adult gut microbiota. The changes that might occur to the function and composition of the adult colonic microbiota as it develops in response to HAMS exposure during weaning needs to be further explored.

Certainly there are inherent limitations associated with *in vitro* fermentation studies and the findings that I have presented will need to be repeated *in vivo* if the potential of HAMS in infant clinical nutrition is to be confirmed. Unfortunately, accepted protocols of how to measure the prebiotic potential of a substrate, particularly in an infant, do not exist. Moreover, dietary interventions conducted during the infant period present unique challenges. Globally, there is significant heterogeneity as to when a child is transitioned to solid foods and the composition of those early food types (Subramanian, Blanton et al. 2015). The introduction to different forms of solids also corresponds to a reduction in the consumption of breast milk and a dramatic change to the

composition of the gut microbiota (Dogra, Sakwinska et al. 2015). These factors could render it difficult to establish a causal relationship between a HAMS intervention conducted during the initiation of complementary feeding, changes to the microbiota, and long-term health outcomes.

Another method to investigate the prebiotic potential of HAMS during infancy could be through the use of gnotobiotic animals (Subramanian, Blanton et al. 2015). HAMS could be administered to gnotobiotic mice harbouring microbiota representative of different stages of infant gut microbial development. The duration of administration could be varied to see if this might have specific effects on the composition of the microbiota.

By examining both taxonomic and functional changes associated with exposure to the RS intervention, predictions may be made regarding the effect of such exposure to the microbiota of the human infant. A similar approach could also be undertaken with gnotobiotic mice colonised with microbiota that is characteristic of severe malnutrition or one that is representative of the gut microbiota post exposure to antibiotics. This use of gnotobiotic mice to identify associations between early diet and changes in the microbiota has precedence. Faith et al introduced a community of human gut bacteria, similar in composition to that of a young infant, into gnotobiotic mice and measured changes in species abundance and microbial gene expression in response to exposure to different dietary ingredients (Faith, McNulty et al. 2011). The authors were able to explain the variation in species abundance by only using their knowledge of the concentrations of particular ingredients present in each meal (Faith, McNulty et al. 2011). Using this method, and by expanding the potential dietary substrates tested, it may be possible to eventually develop a suggested sequence of foods which could be introduced to an infant, factoring in differences in cultural practices, food availability, and affordability (Faith, McNulty et al. 2011). Developing guidelines regarding the timing and composition of solids which are informed by knowledge of the gut microbiota, should be a major priority in public health policy.

Eventually, in order to confirm if HAMS has any role in infant nutrition, an intervention study will need to be conducted in the target population. This study must gather prospective information regarding changes in microbiota composition and diversity, parameters of host inflammation and immune function, markers of fermentation and both short and long term health outcomes. The dose of the prebiotic may affect some of these endpoints (Davis, Martinez et al. 2010) and it could be that a simple feeding study might be needed to determine the optimum dose of HAMS that should be administered during any larger intervention study. This smaller feeding study should also serve to collect information regarding the tolerability, safety and fermentability of a HAMS intervention in an infant population.

Exploring the effects of a HAMS intervention when a person is in good health may guide options for its use in disease states such as environmental enteropathy, acute infectious diarrhoea and of more relevance to high income countries, allergies and asthma. As was discussed in the introduction, several randomised controlled trials have already demonstrated the efficacy of a combined ORS-resistant starch intervention for the treatment of acute infectious diarrhoea in older children and adults (Ramakrishna, Venkataraman et al. 2000; Ramakrishna, Subramanian et al. 2008). Despite major advances in sanitation and interventions such as the ORS, diarrhoeal diseases remain a major cause of global mortality, particularly in young children (Walker, Rudan et al. 2013).

Based on the absence of significant taxonomic change following incubation of the pre-weaning infant faeces with HAMS and the relative reduction in SCFA production when compared to the weaning infants, my *in vitro* fermentation results raise concerns as to whether pre-weaning infants could benefit from the RS component of a combined RS-ORS intervention. Given that the current ORS has no age restriction, establishing the age at which an infant could benefit from such an intervention is of major importance if the combination treatment is to be widely implemented. Whether or not a child has commenced solids may have a greater influence than chronological age on whether they would benefit from an RS-ORS intervention. Interestingly, it is around the time that a child first commences solids that they also appear to be the most vulnerable to diarrhoeal episodes (Walker, Rudan et al. 2013).

In low income countries, on average, a child under the age of five years will experience three diarrhoeal episodes per year (Walker, Rudan et al. 2013). In children, the cumulative effect of repeated episodes of diarrhoea can be profound, resulting in chronic malnutrition, stunting, environmental enteropathy and a reduction in future cognitive capacity (Walker, Rudan et al. 2013). Thus the true extent of the disease burden of diarrhoea reaches far beyond the readily quantifiable mortality figures and strategies to reduce these recurrent attacks, and this must be a global priority.

Following episodes of acute infectious diarrhoea, there is a dramatically altered gut microbial environment, and this along with factors such as the composition of the diet during the recovery period and exposure to antibiotics will influence the dynamics of microbial succession (Lawrence, Ana et al. 2015). This might be an opportunistic time to administer a prebiotic, such as HAMS, to benefit the composition and function of the future resident microbiota. The fermentative capacity of this recovering microbiota has not been well studied, but it may be more favourable. In infants affected by environmental enteropathy, the provision of a fermentable substrate could also contribute to increased energy harvest from the diet through the absorption of colonic SCFAs.

While my *in vitro* fermentation study suggests that HAMS has prebiotic potential in healthy infants, it cannot be assumed that the microbiota of children from other geographical regions with health conditions such as malnutrition and environmental enteropathy will demonstrate a similar capacity. **With this in mind, I was also involved in developing a follow on *in vitro* fermentation study with HAMS which will use the faeces of infants from a low income country (Malawi) with a high prevalence of environmental enteropathy as the initial faecal inocula. Recruitment and collection of samples have already occurred, but the fermentation experiments have yet to be performed.**

In anticipation of my *in vitro* findings being confirmed *in vivo*, several further questions will need to be addressed. For example, would the potential prebiotic and clinical effect of food fortified with HAMS be different from HAMS being supplied as a supplement? Different strategies will have cost and manufacturing implications, and will influence the reach of any HAMS intervention within the target population. Law makers, the agricultural industry and food manufactures have extensive experience the fortification of non-infant foods with HAMS and this expertise will need to be utilised.

It will also be necessary to consider whether the infantile administration of HAMS, be it as a therapeutic or prophylactic intervention, will depend on *a priori* risk assessment. For example, would the benefits be more measurable and of greater clinical consequence, if the intervention was restricted only to particular groups of infants; that is those determined to have a higher than average risk of developing allergies, those most at risk of developing recurrent diarrhoea or perhaps those who had recently received a course of broad spectrum antibiotics.

One major reason for exploring the prebiotic potential of HAMS during infancy, is the increased awareness that prenatal life and early infancy are periods during which crucial body systems are established, including the immune system, gut microbiota, gut barrier integrity, and antigen tolerance. In mice, the provision of the maternal diet with oligosaccharides during pregnancy and lactation, can ameliorate allergic symptoms in the offspring, enhance the regulatory response to allergic inflammation, dampen the Th2 response and alter the offspring's gut microbiota. (Hogenkamp, Knippels et al. 2015; Bouchaud, Castan et al. 2016). It would be very interesting to determine whether a maternal HAMS intervention (during pregnancy and lactation) could achieve such intergenerational change.

In order to resolve these complex questions, rigorously designed, and adequately powered randomized trials of HAMS at different doses and for different durations of time will be needed. Well defined patient improvement outcomes will also need to be measured. Determining how a HAMS intervention compares to a FOS or a GOS based intervention will add value to such research. I am also aware that the manipulation of the emerging microbiota with a resistant starch

intervention may not be without risk of adverse effects. As mentioned in Chapter 4, a recently published study found that the administration of RS to weanling rats was associated with a significant increase in anxiety-like behaviour (Lyte, Chapel et al. 2016). Similar to the results in the infant weaning group, the rodent study also found that the intake of the resistant starch led to an increase in the abundance of Actinobacteria. Given the increased complexity of neuronal activity in humans, modifying and measuring changes in human behaviour following the administration of a dietary prebiotic maybe more problematic.

However the study by Lyte et al, emphasises that the effects of manipulating the microbiota at a vulnerable period of its assembly may lead to unpredicted consequences (Lyte, Chapel et al. 2016). Researchers interested in developing weaning diets high in resistant starch, will need to collect a wide range of clinical data over a sufficient duration of time to ensure that such possible associations are not missed.

7.2 Resistant starch and zinc bioavailability.

Another aim of the thesis was to consider whether resistant starch could also increase the bioavailability of zinc. Zinc deficiency is a major cause of diarrhoea in young children and affects up to two billion people globally (Hotz and Brown 2004). Its prevalence is particularly high among children aged under five years (Wessels and Brown 2012). In children aged >6 months, supplementation of zinc during acute diarrhoea not only reduces the duration and severity of the initial diarrhoea episode, but it also reduces the incidence of diarrhoea in the following months (Walker and Black 2010). Despite WHO recommendations that a 10 day course of oral zinc be provided to children during an episode of acute diarrhoea, the global uptake of therapeutic zinc remains poor (Taneja and Malik 2014).

After confirming that the faecal microbiota of weaning children could ferment HAMS and that it had a prebiotic effect, I considered whether there could be an opportunity to couple the fermentation of HAMS to the absorption of zinc from the colon. Consequently, as part of my thesis, I conducted and published the first review into the potential for the large intestine to participate in zinc absorption. This work was presented in Chapter 5. Essentially, my review found that although absorption of clinically significant zinc from the mammalian colon could occur, there was insufficient data to support such a capacity in the human large intestine. However, I also found that the conditions that might facilitate such absorption had not been adequately explored.

Following the commencement of my PhD, a study was published which found that resistant starch did not enhance the apparent absorption of zinc in stunted, non zinc deficient children (May, Westcott et al. 2015). After reviewing the study findings, I devised a proposal to use a weaning rat model to determine whether preceding zinc deficiency could affect the capacity of a fermentative

substrate to facilitate zinc absorption, a factor not previously addressed in the scientific literature. The results of this study were presented in Chapter 6.

While I confirmed that HAMS could enhance the bioavailability of zinc in rodents, I found that preceding zinc deficiency had no statistical effect on this parameter. Within the boundaries of this thesis and the original aims, I was unable to identify a reason as to why there exists this discrepancy between rodents and humans in relation to the capacity of a fermentable substrate to enhance the absorption of zinc.

The administration of only twelve days of HAMS led to a significant increase in colonic length and caecal size when compared to rats which did not receive the HAMS intervention. Although not measured, it would be reasonable to expect that this increase in potential surface area might have also led to an increase in the number of colonic transporters available to participate in zinc absorption.

A further study could investigate whether the magnitude of this trophic effect of a fermentable substrate on colonic morphology is maintained across the lifespan of the animal. It had previously been shown that the administration of HAMS for 14 days to adult male rats also led to a significant increase in caecal size and a significant reduction in caecal pH(Gee, Faulks et al. 1991). However, due to differences between the studies in relation to the species of rodent and the dose of RS, direct comparison between Gee's study and my study is not possible.

7.2.1 Colonic absorption of minerals in infants

Breastfed infants have lower faecal pH. (Ogawa, Ben et al. 1992) However, there is limited information regarding whether this lower pH could contribute to an increase in capacity for divalent cation absorption from the colon. For example while it is known that the colon can contribute to calcium absorption in adults, there is no comparable information in infants. Comparing the colonic absorption of minerals between weanling and adult rodents might reveal whether such age related differences are worth exploring in humans.

Also emerging from my rodent study was the finding that preceding zinc deficiency had an effect on the fermentation of HAMS. It is possible that these changes were associated with a change in the composition of the gut microbiota associated with the depletion of zinc in the diet. Only a few human studies exist on the effects of host micronutrient deficiency on the host-microbe-metabolic axis and the impact on health. I did not have the resources to investigate the effect of preceding zinc deficiency on gut microbial composition and, subject to funding I would certainly like to examine this further. Zinc is not only essential to humans but it is also essential for many members of the gut microbiota. Depletion of luminal zinc might lead to a less diverse microbial community, one comprised preferentially of species that are more capable of surviving in a zinc

depleted environment. A loss of global microbial diversity may have contributed to the reduction in fermentation capacity that was evident in my study.

In a study conducted before the advent of molecular sequencing, it was demonstrated that conventionally raised mice require twice as much dietary Zn as their germ-free counterparts (Smith, McDaniel et al. 1972). Thus, the intake of zinc must be adequate enough to satisfy the requirements of not only the host but also the resident microbiota. It would be reasonable to expect competition between members of the gut microbiota for luminal zinc when the zinc intake is low. Under such conditions of dietary zinc deficiency, there may be a selective advantage for those species that are able to survive at low-Zn levels. To my knowledge, there are no studies examining the effect of dietary zinc depletion or supplementation on the composition or function of the gut microbiome in humans.

The few animal studies that have been published in this field are informative. In a study conducted in broilers, it was demonstrated that chronic zinc deficiency can lead to a significant reduction in species richness and species diversity (Reed, Neuman et al. 2015). In animals with zinc deficiency, there was an expansion of the phylum Proteobacteria, as well as the genera Enterobacteriaceae and Enterococcus (Reed, Neuman et al. 2015). One explanation for these findings could be that under Zn limiting conditions, the high affinity Zn transporter, ZnuABC is up-regulated within these bacterial groups, offering them a selective advantage when the concentration of luminal Zn is low (Hantke 2001; Gielda and DiRita 2012).

Pieper et al demonstrated that zinc supplementation in weaning piglets had a dose response effect on ileal bacterial community composition and activity (Pieper, Vahjen et al. 2012). They found that species richness and Shannon diversity were significantly increased at higher levels of zinc supplementation (Pieper, Vahjen et al. 2012). Such a finding could have translational implications for human health. In another study, the provision of high dietary zinc was found to have both transient and lasting effects on the development of the intestinal microbiota, affecting both composition and metabolic activity (Starke, Pieper et al. 2014). It follows that the effect of dietary zinc deficiency or supplementation on the emerging gut microbiota of a human infant may be very different to the effects of the same intervention on an adult gut microbiota. This need to study the effects of an intervention across different age ranges and for the duration of the study to be sufficient to make long term predictions on the composition, function and clinical relevance of any sustained changes in the microbiota, highlights some of the challenges associated with human gut microbiota research.

It is possible that a change in the composition and function of the microbiota in response to the initial dietary zinc depletion negated the effect of any concurrent increase in the expression of colonic zinc transporters. For example, upon the reintroduction of zinc to the diet there might have

been an increase in microbial sequestration of the luminal zinc and the relative reduction in caecal SCFAs following the provision of HAMS to these zinc deficient rats might also have compromised colonic zinc bioavailability (Reed, Neuman et al. 2015).

Regardless of the exact mechanisms, I could not demonstrate that preceding zinc deficiency enhanced the effect of a fermentable substrate on zinc status. Consequently, I cannot recommend investing further resources into examining whether administering a fermentable substrate such as HAMS to infants with preceding zinc deficiency, might reveal a latent capacity for the human colon to participate in zinc absorption.

7.3 Conclusion

The initial aims of this thesis have been satisfied. I have demonstrated that the faecal inocula of pre-weaning infants has some capacity to ferment HAMS, but this capacity is significantly increased post the commencement of solids. I have confirmed that a commercially available mHAMS can also be fermented by the faecal inocula of weaning infants. Most significantly, for the first time, I have demonstrated the prebiotic potential of both of these starches in an infant population. Expanding the list of recognised infant prebiotics to include HAMS will now depend on further *in vivo* research. A further aim of the thesis was to consider whether the fermentation of HAMS could be coupled to the absorption of zinc – a highly relevant issue if HAMS is to have a wider role in the treatment of acute childhood diarrhoea.

This required a review of the colon's capacity to absorb zinc and, based on this work, I found that the capacity of preceding zinc deficiency to influence the colonic absorption of zinc had not been previously considered. Using an animal model, I examined this relationship, and while confirming that zinc status was enhanced by dietary administration of HAMS, I found that preceding zinc status did not influence this effect. However, I also demonstrated that preceding zinc deficiency has a profound effect on the fermentation of HAMS. Further research into the effects of host micronutrient intake on gut microbial composition and function is now required.

Chapter 8 Appendix

8.1 Ethics Approval Letter

20 September 2013

Professor Graeme Young
L3 / Flinders Centre for Innovation in Cancer
BEDFORD PARK SA 5042

Dear Professor Young

HREC reference number: HREC/13/SAC/205 (339.13)
SSA reference number: SSA/13 /SAC/302
Project title: Fermentation of type 2 and type 4 resistant starch by infant faecal bacteria pre and post weaning.
Ethics approval: 06 September 2013 to 06 September 2016

RE: Site Specific Assessment Review

Thank you for submitting an application for authorisation of this project. I am pleased to inform you that authorisation has been granted for this study to commence at the following site: Flinders Medical Centre.

- Site Specific Assessment form
- CV for Professor Graeme Young
- Letter from John Markic, Manager Insurance Services SA Health, advising the indemnity and insurance being provided by Flinders University is acceptable dated 24 April 2013
- Advertisement dated 26 August 2013
- Third party consent form dated 26 August 2013
- Parent/Guardian participant information sheet dated 26 August 2013
- Adult volunteers participant information sheet and consent form dated 26 August 2013

Should you have any queries about the consideration of your Site Specific Assessment form, please contact Bev Stewart Campbell on 08 8204 4507.

The SSA reference number should be quoted in any correspondence about this matter.

Yours sincerely

Bev Stewart Campbell
Research Governance Officer
Southern Adelaide Clinical Human Research Ethics Committee

Flinders Medical Centre
The Flats G5 – Rooms 3 and 4
Flinders Drive, Bedford Park SA 5042
T: 08 8204 6453
F: 08 8204 4586
E:Research.ethics @health.sa.gov.au

8.2 Participant Information Sheet

Name of Organisation

Flinders University of SA, Global Gastrointestinal Health

Parents and guardians

Title of the project

Fermentation of type 2 and type 4 resistant starch by infant faecal inocula

Lay title

Use of infant stool to predict if starch can offer a health benefit to young children.

Researchers

Dr Geetha Gopalsamy (Phd student), Global Gastrointestinal Health, FCIC, Flinders University.

Prof Graeme Young Head of Global Gastrointestinal Health, FCIC, Flinders University.

(Supervisor of Dr Geetha Gopalsamy's PhD)

Invitation to participate

You are invited to participate in a research project that will use two separate specimens of your infant's stool to find out if they have acquired the bowel organisms needed to ferment resistant starch. Whether you wish to participate in provision of these samples is entirely up to you. Whether you and your child take part or not, your child's medical care/relationship with the services they receive will not be affected in any way.

Aims of the project

Resistant starch, a special type of dietary starch, could help treat severe dehydration during acute gastroenteritis in children. This project will use **two stool samples** from your infant to find out if their gut has acquired the necessary bacteria to digest resistant starch. Without this digestion process, resistant starch will be of no health benefit. Many children around the world, millions in total, suffer severely from gastroenteritis and we need to find out if infants acquire the necessary bacteria to digest RS before they are weaned. This will help us devise strategies to reduce the number of children who suffer.

Summary of procedures

Your child will **not** be involved in any uncomfortable procedure or any procedure that could affect their health.

We are seeking suitable infants to provide two fresh stool specimens approximately 4 months apart. (pre and post commencement of solid foods.). Stool will need to be collected from your child's potty or nappy and be transferred to the specimen container provided within 15 minutes. This container should then be placed into the zip lock bag provided. This bag should then be kept in the provided freezer until collected by the courier. The specimen needs to be processed within 4 hours of passage of the stool.

A short questionnaire will also need to be completed. We will need to know age of your child, whether they were delivered by caesarean section (as this may affect the type of bacteria in their gut) and whether they were exclusively breast or formula fed.

After providing the initial stool sample, we will ask you to contact us once your child has commenced eating mashed foods. If we have not heard from you 4 months after your first sample we will contact you again.

Commitments

You will be asked to provide two fresh stool specimen from your infant over a 4 month period. Each specimen needs to be received at our laboratory **within 4 hours** of being collected. It will then be processed in the laboratory and frozen until analysed. We can provide a portable freezer which can be stored at your house until the time a stool sample can be obtained.

We will provide you with the telephone number of a specific courier or research team member who can be contacted to collect the specimen. Given the time requirements we will provide a schedule of times for pick up.

If for some reason, the stool specimen is insufficient or received outside of the recommended time then a replacement specimen maybe requested.

Benefits

While your child may not directly benefit from participating in this study, the overall study findings may have real implications for improving infant health worldwide. If fecal inocula of children less than 1 is shown to have the capacity to digest resistant starch, then such starches could be used in infant food formulations to provide various important health benefits, including treatment of dehydration during acute childhood diarrhoea or as a prebiotic, promoting the growth of healthy gut bacteria in young children.

Risks and adverse effects

There are no anticipated risks from participating in this research project.

Compensation

If you or your child suffer injury as a result of participation in this research or study, compensation might be paid without litigation. However, such compensation is not automatic and you may have to take legal action to determine whether you should be paid.

Confidentiality

All records containing personal information will remain confidential and no information which could lead to your identification will be released, except as required by law.

Publication

The results once analysed may subsequently be published in an international scientific journal. You or your child will not be personally identified in any report or publication. These results will be available to you when the study has been completed and all the analysis is finalized.

Withdrawal

Your participation in this study is entirely voluntary and you have the right to withdraw your child from the study at any time without giving a reason. If you decide not to participate in this study, or if you withdraw from the study, you may do so freely, without affecting the standard care that your child will receive.

Outcomes

If you would like to be informed of the results of the study, we can provide a letter describing the outcomes from the study once all data is analysed.

Expenses and payments

You will not receive any payment for participation in this study apart from compensation for reasonable travel costs for visits to deliver the fecal specimens.

Statement about incentives for the researcher

You should be aware that your doctor and/or research team receive no financial benefit from enrolling you in this study.

Statement of confidentiality

All records containing personal information will remain confidential and no information that could lead to your identification will be released, except as required by law

Storage of samples and future use of samples:

Following the completion of this study, remaining stool samples will be stored in case of future assessment of the microbial population is required to interpret results. Where samples are not used for this purpose they will be destroyed within a 2 year period of study completion.

Contact

Should you require further details about the project, either before, during or after the study, you may contact the Dr Geetha Gopalsamy,0401006607 Bedford Park SA 5042

Complaints

This study has been reviewed by the Southern Adelaide Clinical Human Research Ethics Committee. If you wish to discuss the study with someone not directly involved, in particular in relation to policies, your rights as a participant, or should you wish to make a confidential complaint, you may contact the Executive Officer on 8204 6453 or email research.ethics@health.sa.gov.au

8.3 Instruction for Faecal Sample Collection – Infants

For the purposes of the study it is important that all specimens are uncontaminated. Please follow the following directions.

On the nominated day a portable WAECO freezer will be delivered to your house. Please plug the freezer into the power point and switch on. Ensure that temperature is set to -18 and freezer is working.

A good place for the freezer may be the garage, away from any young, inquisitive children.

Collecting the specimen:

As soon as you recognise that your baby has passed a bowel motion, please use the lid from the provided brown container to scoop up as large a sample of stool from the nappy into the container. After screwing the lid closed, please place the container into the provided zip locked bag. Place this bag (containing the stool sample) into the larger bag and secure with the zip tie. Place this bag into the provided freezer. Then call the courier to arrange for the freezer to be collected.

Please note that to ensure the accuracy of the study the stool specimen needs to be processed in the laboratory within **4 hours of being collected**.

The freezer can remain with you until a suitable sample has been collected.

Times for freezer collection:

MONDAY, TUESDAY, FRIDAY: anytime between 8-4 pm

WED,THUR: anytime between 8-11am

Please keep in mind that closer the collection of the sample is to the actual passage of the stool, the more accurate the results will be

Thank you for volunteering for this study. Your time is greatly appreciated.

8.4 Methodology for separation and visualisation of PCR products for analysis using agarose gel matrix

Gel electrophoresis is a method for separating a mixed population of DNA fragments by length, enabling subsequent analysis or isolation of the separated DNA. Separation is achieved by applying an electric field to move the negatively charged molecules through an agarose matrix. The shorter the DNA fragment, the faster and further it will migrate along the pores of the gel.

Equipment

- Electrophoresis tank, casting trays, well combs, frozen metal bar,
- Power supply

Reagents

- Milli-Q water
- 50x TAE buffer
- DNA marker (Hyperladder VI) Bioline
- Agarose powder 1.5% agarose concentration (dissolve 1.5g agarose powder in 100mL of 0.5x TAE buffer in Schott bottle)
- Gel Green Stain 10,000X in water Jomar Bioscience
- 6x Gel Loading Buffer 0.25 mL of 2% bromophenol blue, 0.25mL of 2% Xylene Cyanol, 2.5 mL milliQ water and 7.0 mL of 100% Glycerol to 10 mL beaker.

Procedure

Preparation and pouring of agarose gel

Melt prepared agarose gel mix in microwave. Allow gel to cool to approximately 60^0C. Whilst cooling add 10uL of Gel Green Stain per 100mL of agarose mix. Place white bar at open end of casting tray. Position well combs. Place frozen metal bar behind and touching the white bar to assist in setting the agarose and forming a seal at end of tray. Pour agarose into gel casting tray and allow gel to set at room temperature.

Preparation of running system

Remove metal bar when gel is set. Place gel (in casting tray into gel electrophoresis tank. Pour 0.5 X TAE buffer into each end of tank, ensuring horizontal metal bars in tank completely submerged in buffer. Pour distilled water on top of gel then remove combs.

Preparing and loading samples

Add 1 uL of 6xgel loading buffer and 5uL of sample into each well (mix together first) Load 5uL marker in first well (Easy Ladder 1). Load samples into wells. Place lid on tank. Connect electrodes into power pack and run samples between 200-220 Volts. When loading buffer reaches ¾ way down gel turn power off and remove casting tray and gel from the tank.

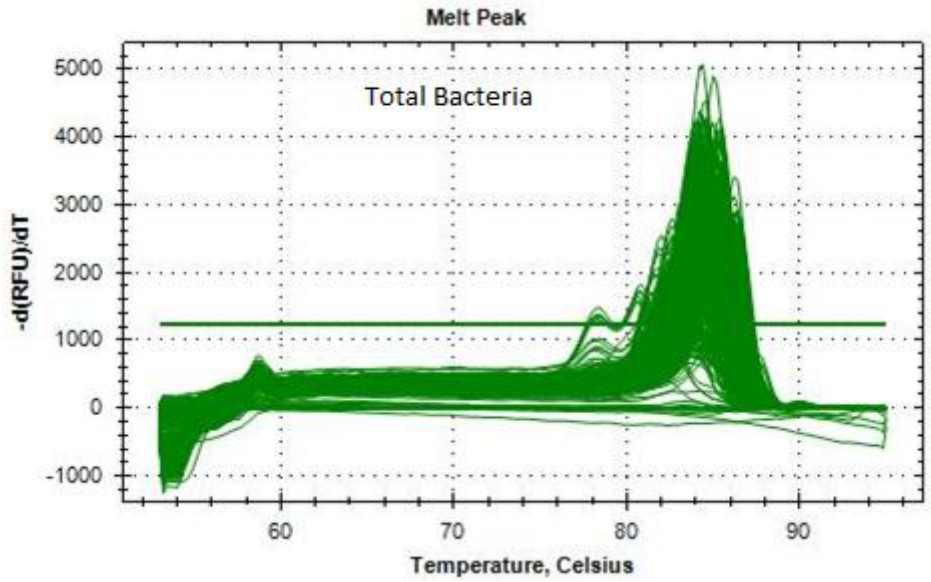
Viewing the gel

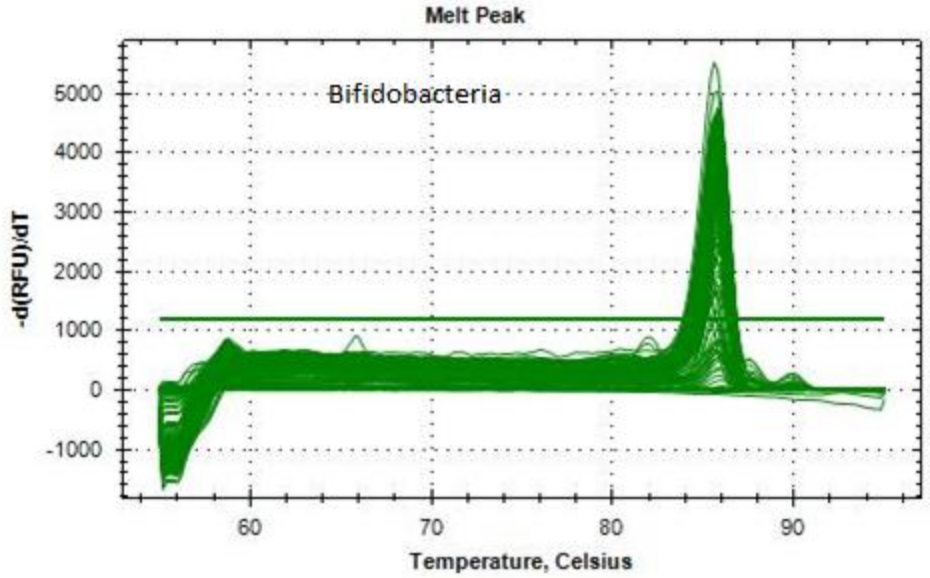
Transfer the gel in tray into dark room. Remove gel from casting tray onto transilluminator. Turn lights off and put on orange glasses to visualise glowing bands on gel. Capture image using computer Nikon Capture software.

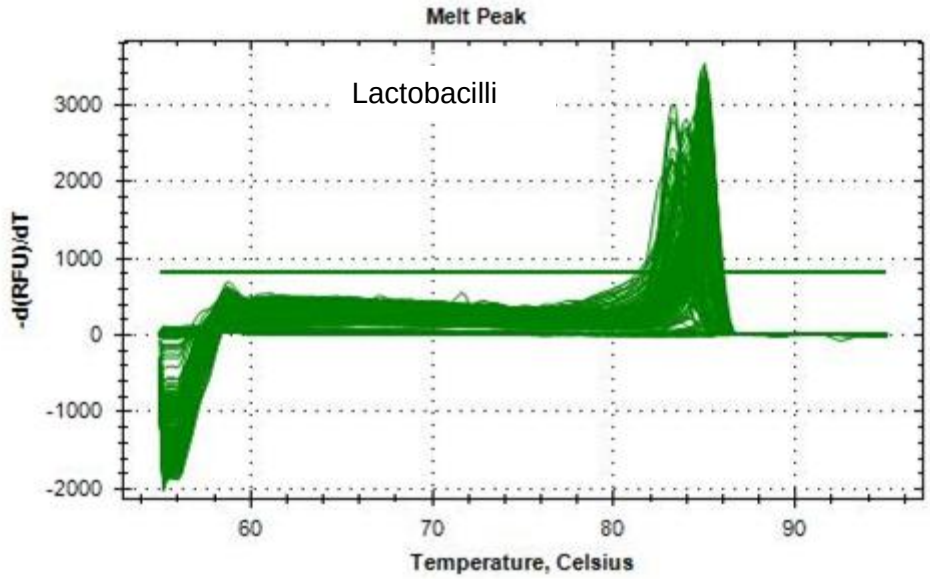
8.5 Purification of DNA fragments from Agarose Gel utilising Roche DNA extraction kit.

Place excised agarose gel into 1.5mL sterile microcentrifuge tube. Add 300uL Binding buffer for every 100mg agarose gel slice to microcentrifuge tube. Vortex tube 15-30 s to resuspend gel slice in Binding buffer. Incubate suspension for 10 minutes at 56^{0}C. Once agarose gel has dissolved add 150uL isopropanolol for every 100mg agarose gel slice in tube. Insert one High Pure Filter Tube into one Collection Tube. Pipette contents of microcentrifuge into upper reservoir of Filter tube. Centrifuge at maximum speed at room temperature on table top centrifuge for 60 seconds.. Discard flowthrough solution. Reconnect filter tube with same collection tube. Add 500 uL of wash buffer to upper reservoir and again centrifuge for 1 minute at maximum speed. Discard flowthrough solution. Recombine Filter Tube with same Collection Tube, then add 200 uL Wash Buffer. Centrifuge for 1 minute at maximum speed. Discard flowthrough solution and Collection tube. Recombine Filter Tube with a clean 1.5mL microcentrifuge tube. Add 50-100uL elution Buffer to upper reservoir of the Filter tube. Centrifuge 1 min at maximum speed. Microcentrifuge tube now contains purified DNA. Store eluted DNA at -20^{0}C for later analysis.

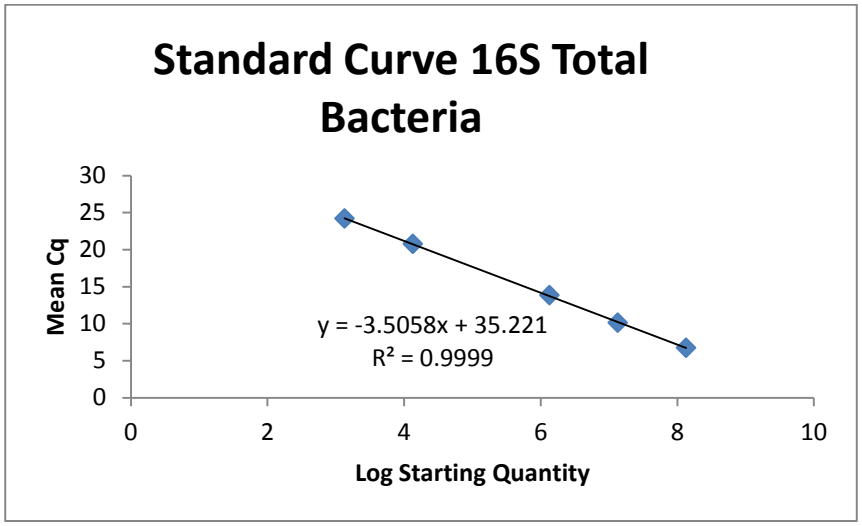
8.6 Melting Curves for total Bacteria, Bifidobacteria and Lactobacilli.

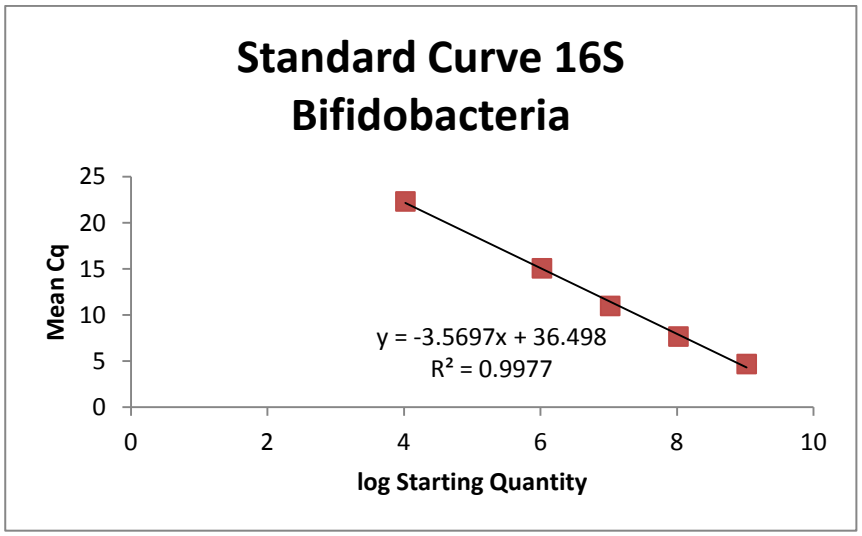


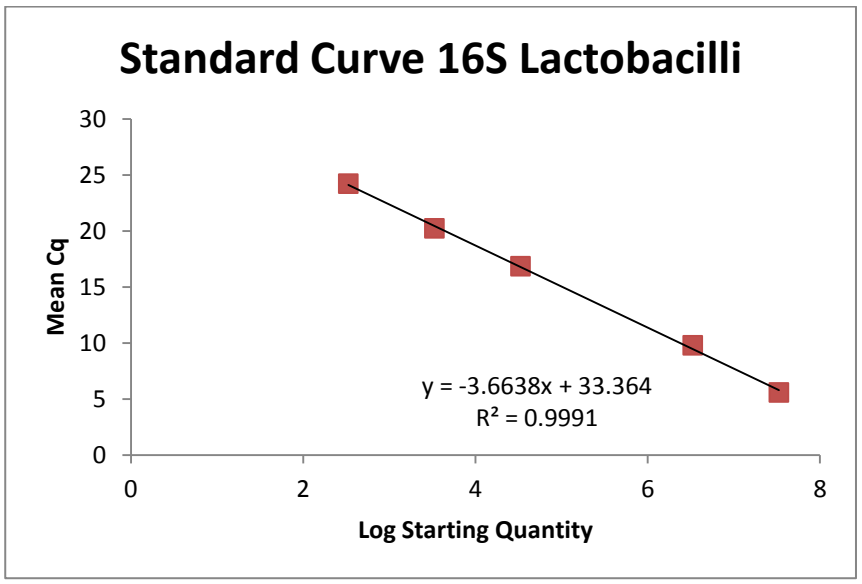

Melt Peak
Bifidobacteria
-d(RFU)/dT
5000
4000
3000
2000
1000
0
-1000
60
70
80
90
Temperature, Celsius


Melt Peak
Lactobacilli
-d(RFU)/dT
3000
2000
1000
0
-1000
-2000
60
70
80
90
Temperature, Celsius

8.7 Standard Curves



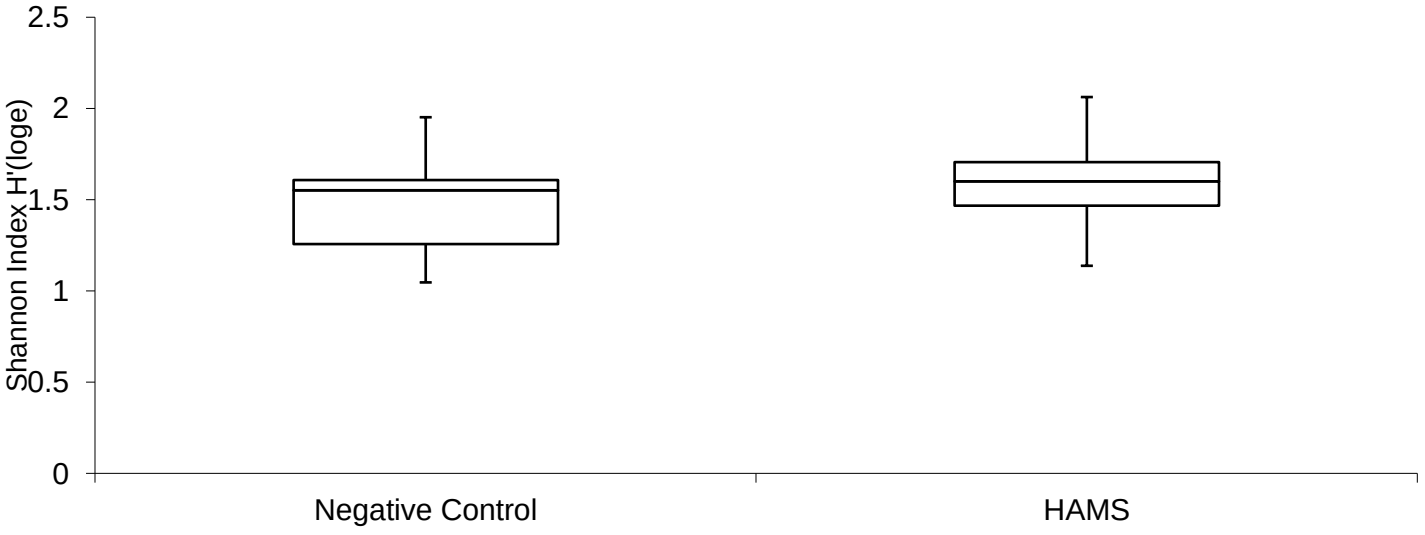




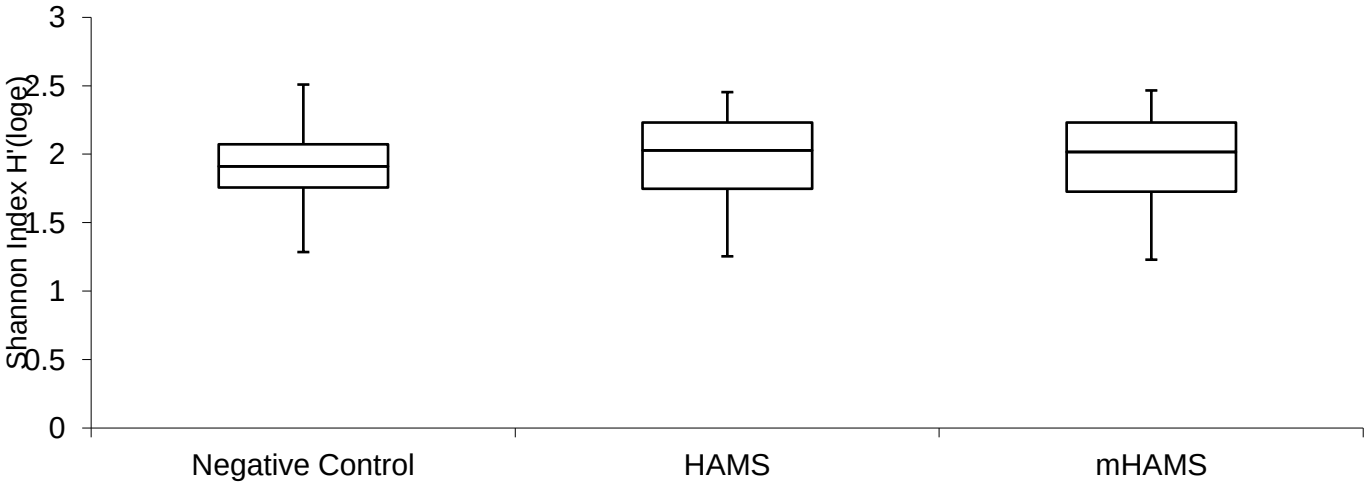
8.8 Shannon's Index for calculation of alpha diversity

Within sample diversity at the **genus level** was estimated using the Shannon index (loge). A one way ANOVA was used to determine if there was any difference in the diversity of the bacterial communities following incubation with the different substrates. In the pre-weaning group the overall mean of the log(e) of Shannon's index did not differ between the negative control and HAMS incubations (F = 2.06,df=1, p=.16). In the weaning group there was also no difference in the log(e) of Shannon's index according to treatment group (F=.51, df=2, p=.601). Boxes indicate 25th to 75th percentiles, with mean values marked as a line and whiskers indicating minimum and maximum values.

Pre-weaning (genus)

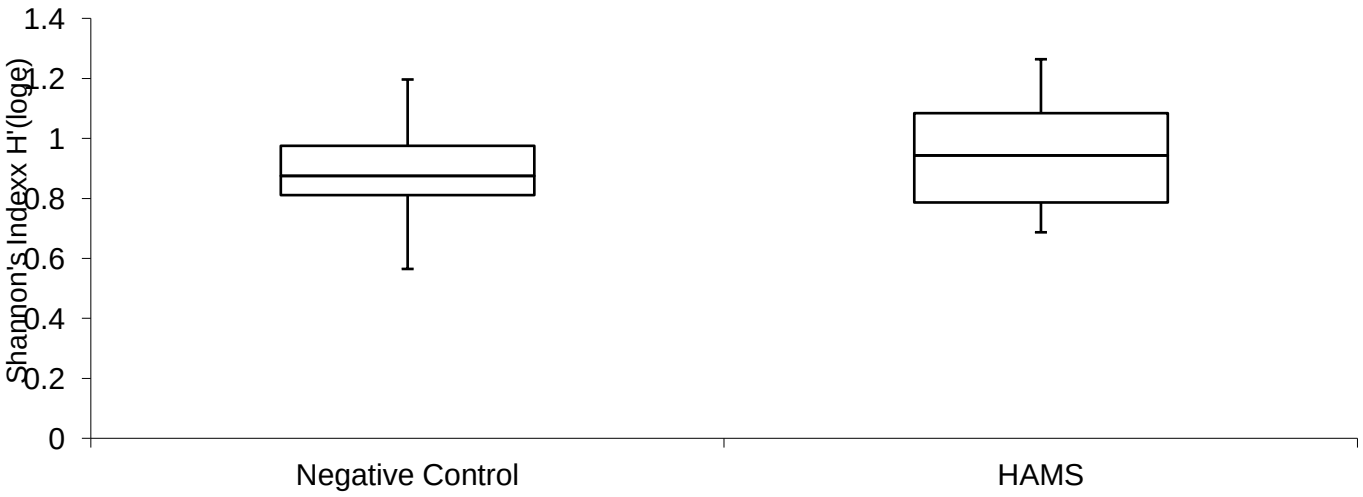


Weaning (genus)

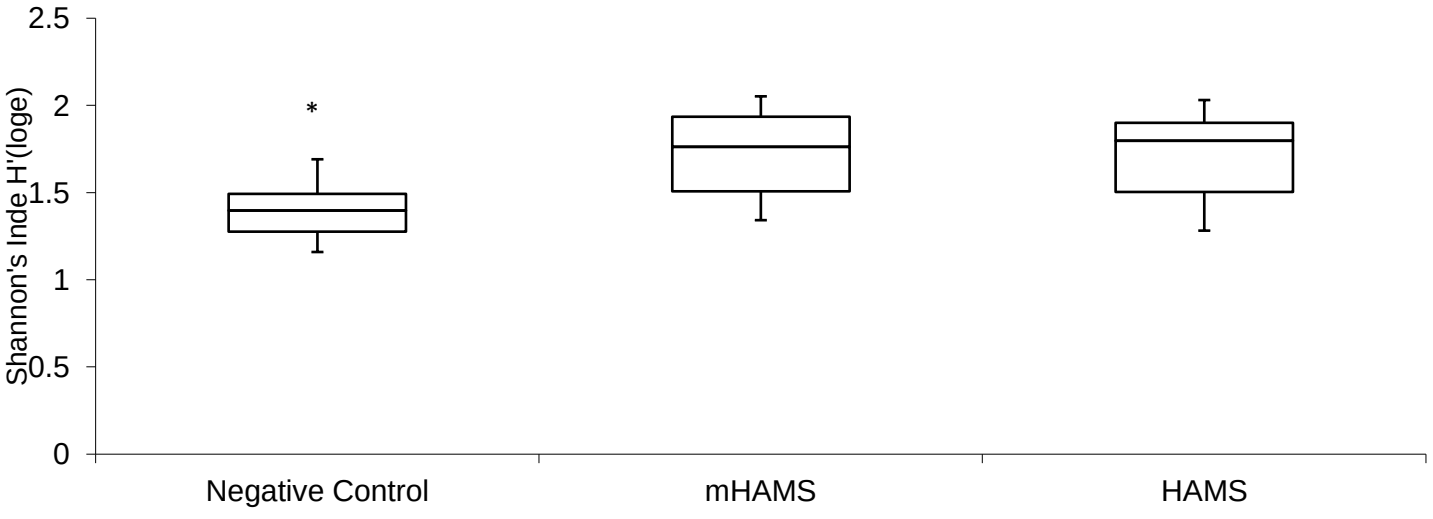


Within sample diversity at the **phylum level** was estimated using the Shannon index (loge). A one way ANOVA was used to determine if there was any difference in the diversity of the bacterial communities following incubation with the different substrates. In the pre-weaning group the overall mean of the log(e) of Shannon's index did not differ between the negative control and HAMS incubations (F = 4.20,df=1, p=.23). In the weaning group the one way ANOVA was significant (F=3.22, df=2, p<0.05). Post hoc comparison using t tests with bonferroni correction indicated that the mean H'loge for the negative control incubations (M=1.43, SD=0.16) was significantly different than for the mHAMS (M =1.7, SD =.24) and HAMS (1.69, SD.25) incubations. However the mean H'loge for mHAMS and HAMS did not significantly differ.

Pre-weaning - phylum



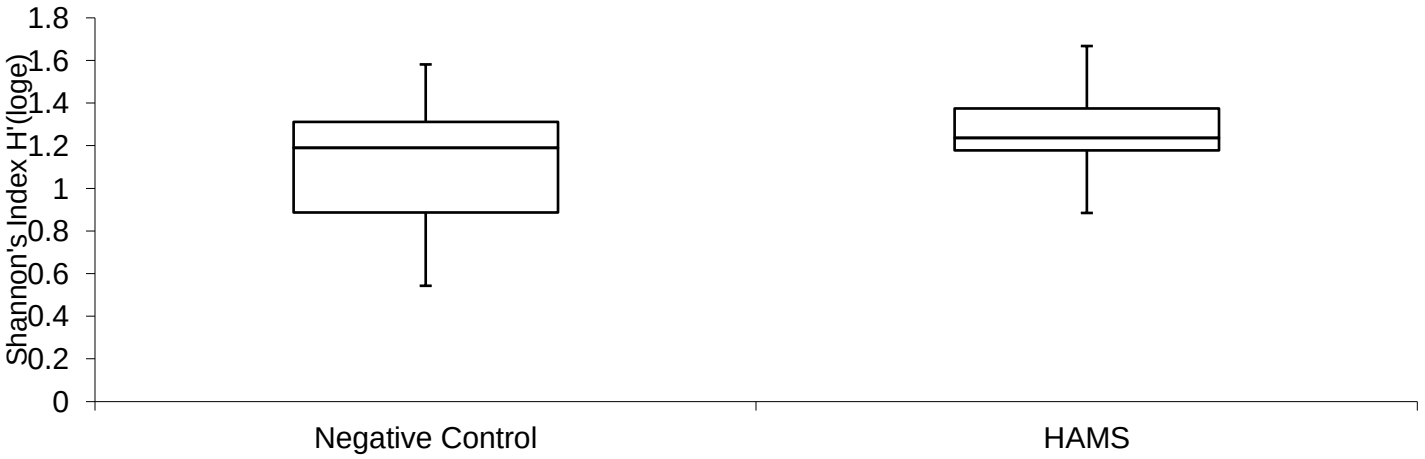
Weaning (phylum)



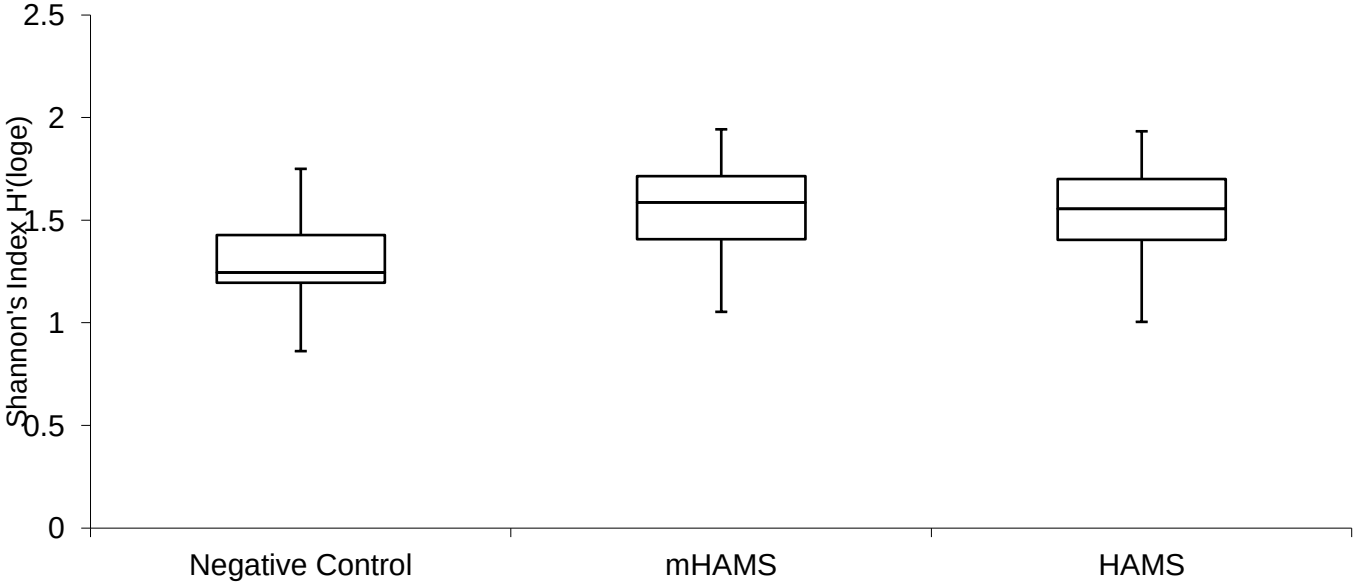
Within sample diversity at the **order level** was estimated using the Shannon index (loge). A one

way ANOVA was used to determine if there was any difference in the diversity of the bacterial communities following incubation with the different substrates. In the pre-weaning group the overall mean of the log(e) of Shannon's index did not differ between the negative control and HAMS incubations (F = 2.37,df=1, p=.14). In the weaning group the one way ANOVA was significant (F=5.05, df=2, p<0.05). Post hoc comparison using t tests with bonferroni correction indicated that the mean H'loge for the negative control incubations (M=1.3, SD=0.24) was significantly different than for the mHAMS (M =1.56, SD =.27) and HAMS (1.54, SD=.25) incubations. However the mean H'loge for mHAMS and HAMS did not significantly differ.

Pre-weaning (order)

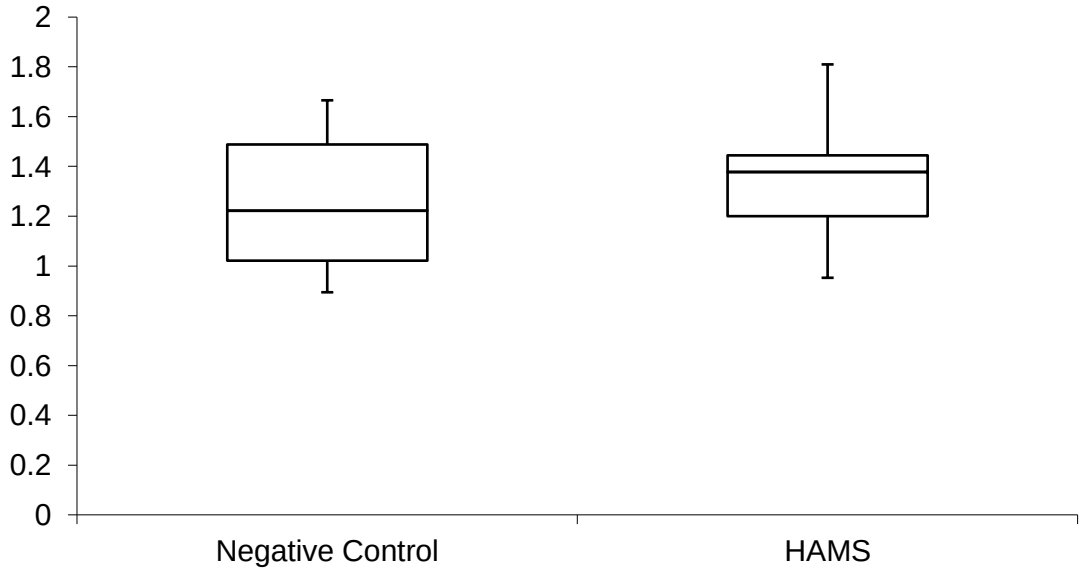


Weaning (order)

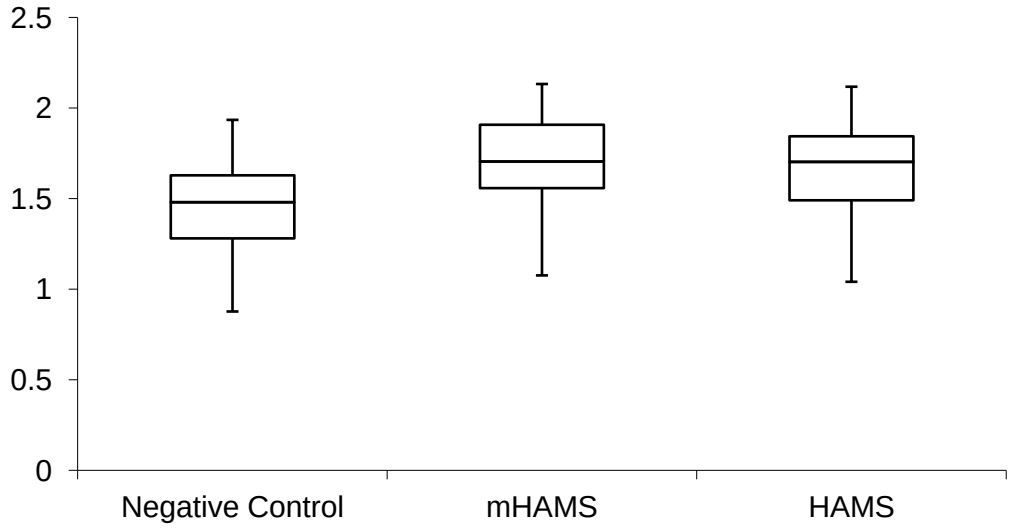


Within sample diversity at the **family level** was estimated using the Shannon index (loge). A one way ANOVA was used to determine if there was any difference in the diversity of the bacterial communities following incubation with the different substrates. In the pre-weaning group the overall mean of the log(e) of Shannon's index did not differ between the negative control and HAMS incubations (F =1.05,df=1, p=.31).). In the weaning group there was also no difference in the loge of Shannon's index according to treatment group (F=3.07, df=2, p=.06)

Pre-weaning (family)

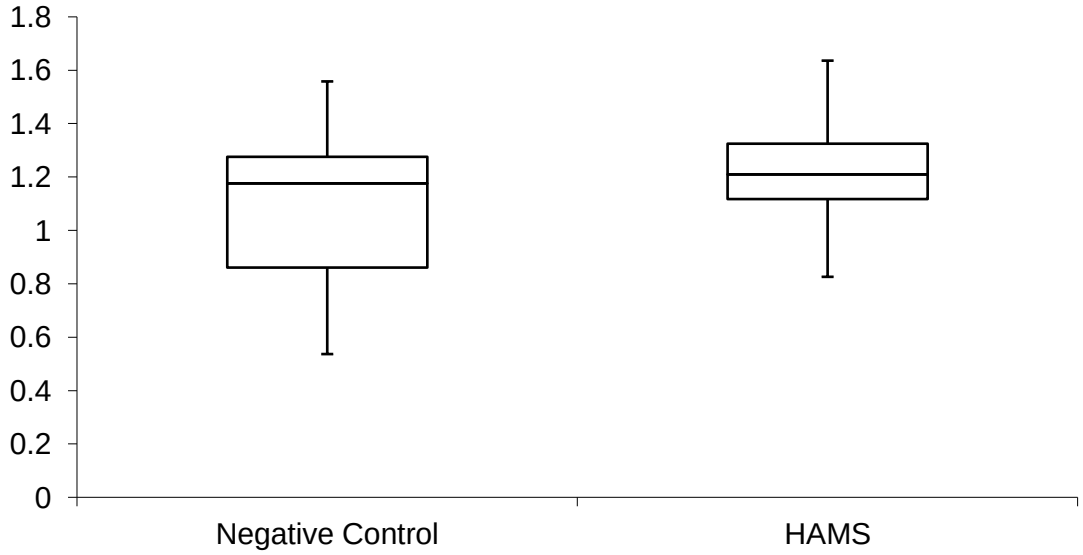


Weaning (family)

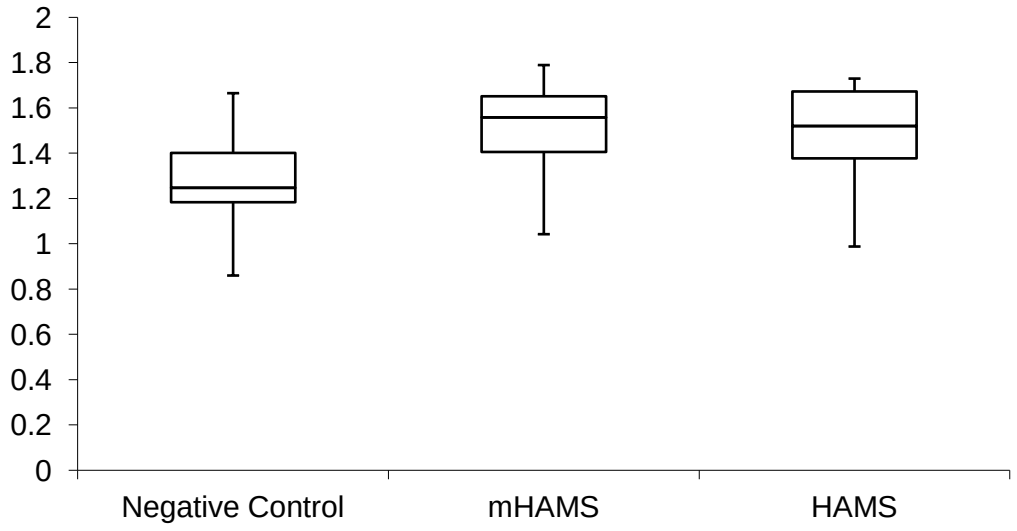


Within sample diversity at the **class level** was estimated using the Shannon index (loge). A one way ANOVA was used to determine if there was any difference in the diversity of the bacterial communities following incubation with the different substrates. In the pre-weaning group the overall mean of the log(e) of Shannon's index did not differ between the negative control and HAMS incubations (F = 2.80,df=1, p=.10). In the weaning group the one way ANOVA was significant (F=4.76, df=2, p=0.014). Post hoc comparison using t tests with bonferroni correction indicated that the mean H'loge for the negative control incubations (M=1.28, SD=0.22) was significantly different than for the mHAMS (M =1.51, SD =.23) and HAMS (1.48, SD=.22) incubations. However the mean H'loge for mHAMS and HAMS did not significantly differ.

Pre-weaning (class)



Weaning (class)



8.9 Allocation of rats into groups, based on initial body weight

Zinc Deficient/RS	Zinc Deficient/CS	Zinc Replete/RS	Zinc Replet/CS
96	94	108	101
102	105	102	95
97	103	96	104
100	96	98	98
97	104	109	97
102	94	96	96
99	100	105	103
98	102	95	105

Groups	*Count*	*Sum*	*Average*	*Variance*
Group 1	8	791	98.875	5.267857
Group 2	8	798	99.75	20.21429
Group 3	8	809	101.125	32.125
Group 4	8	799	99.875	14.98214

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	20.59375	3	6.864583	0.37827	0.769373	2.946685
Within Groups	508.125	28	18.14732			

References

Abell, G. C. J., C. M. Cooke, et al. (2008). "Phylotypes related to Ruminococcus bromii are abundant in the large bowel of humans and increase in response to a diet high in resistant starch." FEMS Microbiology Ecology **66**(3): 505-515.

Aggett, P. (2010). "Premastication." Matern Child Nutr **6**(1): 2-3.

Aguirre, M., A. Eck, et al. (2015). "Evaluation of an optimal preparation of human standardized fecal inocula for in vitro fermentation studies." J Microbiol Methods **117**: 78-84.

Alam, N. H., H. Ashraf, et al. (2015). "Efficacy of partially hydrolyzed guar gum (PHGG) supplemented modified oral rehydration solution in the treatment of severely malnourished children with watery diarrhoea: a randomised double-blind controlled trial." Journal of Health, Population and Nutrition **34**(1): 1-9.

Andrew, L. K., P. A. Philip, et al. (2011). "Human nutrition, the gut microbiome and the immune system." Nature **474**(7351): 327-336.

Andrews, G. K. (2008). "Regulation and function of Zip4, the acrodermatitis enteropathica gene." Biochem Soc Trans **36**(Pt 6): 1242-1246.

Annison, F., R. Illman, et al. (2003). "Acetylated propionylated or butyralated starches raise large bowel short chain fatty acids preferentially when fed to rats." Nutrient metabolism **133**(11): 3523-3528.

Argenzio, R. A., H. W. Moon, et al. (1984). "Colonic compensation in transmissible gastroenteritis of swine." Gastroenterology **86**(6): 1501-1509.

Armstrong, E., M. Eastwood, et al. (1992). "The effect of weaning diet on the subsequent colonic metabolism of dietary fibre in the adult rat." British Journal of Nutrition **68**: 741-751.

Arpaia, N. (2014). "Keeping peace with the microbiome: acetate dampens inflammatory cytokine production in intestinal epithelial cells." Immunol Cell Biol **92**(7): 561-562.

Arslanoglu, S., G. E. Moro, et al. (2007). "Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life." J Nutr **137**(11): 2420-2424.

Arslanoglu, S., G. E. Moro, et al. (2008). "Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life." J Nutr **138**.

Aryana, K., F. Greenway, et al. (2015). "A resistant-starch enriched yogurt: fermentability, sensory characteristics, and a pilot study in children." F1000Research **4**: 139.

Ashwar, B. A., A. Gani, et al. (2016). "Preparation, health benefits and applications of resistant starch—a review." Starch - Stärke **68**(3-4): 287-301.

Avershina, E., O. Storro, et al. (2014). "Major faecal microbiota shifts in composition and diversity with age in a geographically restricted cohort of mothers and their children." FEMS Microbiol Ecol **87**(1): 280-290.

Bajka, B. H., D. L. Topping, et al. (2006). "Butyrylated starch is less susceptible to enzymic hydrolysis and increases large-bowel butyrate more than high-amylose maize starch in the rat." Br J Nutr **96**(2): 276-282.

Barry, J. L., C. Hoebler, et al. (1995). "Estimation of the fermentability of dietary fibre in vitro: a European interlaboratory study." British Journal of Nutrition **74**(3): 303-322.

Bergstrom, A., T. H. Skov, et al. (2014). "Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants." Appl Environ Microbiol **80**(9): 2889-2900.

Bermudez-Brito, M., C. Rosch, et al. (2015). "Resistant starches differentially stimulate toll-like receptors and attenuate proinflammatory cytokines in dendritic cells by modulation of intestinal epithelial cells." Mol Nutr Food Res.

Bertelsen, R. J., E. T. Jensen, et al. (2016). "Use of probiotics and prebiotics in infant feeding." Best Practice & Research Clinical Gastroenterology **30**(1): 39-48.

Bhattacharya, S. K. (2000). "Therapeutic methods for diarrhea in children." World Journal of Gastroenterology **6**(4): 497-500.

Bindels, L. B., N. M. Delzenne, et al. (2015). "Towards a more comprehensive concept for

prebiotics." Nat Rev Gastroenterol Hepatol **12**(5): 303-310.
Binder, H. (2010). "Role of colonic short chain fatty acid transport in diarrhea." Annu Rev Physiol. **72**: 297-313.
Binder, H. J., I. Brown, et al. (2014). "Oral rehydration therapy in the second decade of the twenty-first century." Curr Gastroenterol Rep **16**(3): 376.
Binder, H. J. and P. Mehta (1989). "Short-chain fatty acids stimulate active sodium and chloride absorption in vitro in the rat distal colon." Gastroenteroloy **96**.
Binns, C. W., A. H. Lee, et al. (2007). "The CUPDAY Study: prebiotic-probiotic milk product in 1–3-year-old children attending childcare centres." Acta Pædiatrica **96**(11): 1646-1650.
Bird, A. R., I. L. Brown, et al. (2000). "Starches, resistant starches, the gut microflora and human health." Curr Issues Intest Microbiol **1**(1): 25-37.
Bird, A. R., M. A. Conlon, et al. (2010). "Resistant starch, large bowel fermentation and a broader perspective of prebiotics and probiotics." Beneficial Microbes **1**(4): 423-431.
Bird, A. R., S. Usher, et al. (2012). Resistant Starch. Dietary Fiber and Health, CRC Press**:** 41-56.
Birkett, A. M., J. C. Mathers, et al. (2000). "Changes to the quantity and processing of starchy foods in a western diet can increase polysaccharides escaping digestion and improve in vitro fermentation variables." Br J Nutr **84**(1): 63-72.
Bischoff, S. C., G. Barbara, et al. (2014). "Intestinal permeability - a new target for disease prevention and therapy." Bmc Gastroenterology **14**.
Bjorksten, B., P. Naaber, et al. (1999). "The intestinal microflora in allergic Estonian and Swedish 2-year-old children." Clin Exp Allergy **29**(3): 342-346.
Bjorksten, B., E. Sepp, et al. (2001). "Allergy development and the intestinal microflora during the first year of life." J Allergy Clin Immunol **108**(4): 516-520.
Bodinham, C. L., L. Smith, et al. (2014). "Efficacy of increased resistant starch consumption in human type 2 diabetes." Endocrine Connections **3**(2): 75-84.
Boehm, G. and G. Moro (2008). "Structural and functional aspects of prebiotics used in infant nutrition." J Nutr **138**(9): 1818S-1828S.
Bondue, P. and V. Delcenserie (2015). "Genome of Bifidobacteria and Carbohydrate Metabolism." Korean Journal for Food Science of Animal Resources **35**(1): 1-9.
Bouchaud, G., L. Castan, et al. (2016). "Maternal exposure to GOS/inulin mixture prevents food allergies and promotes tolerance in offspring in mice." Allergy **71**(1): 68-76.
Bougle, D., N. Vaghefi-Vaezzadeh, et al. (2002). "Influence of short-chain fatty acids on iron absorption by proximal colon." Scand J Gastroenterol **37**(9): 1008-1011.
Bourquin, L. D., E. C. Titgemeyer, et al. (1996). "Fermentation of various dietary fiber sources by human fecal bacteria." Nutrition Research **16**(7): 1119-1131.
Boyle, R., N. Brown, et al. (2015). "Partially hydrolysed, prebiotic supplemented whey formula for the prevention of allergic manifestations in high risk infants: a multicentre double-blind randomised controlled trial." Clinical and Translational Allergy **5**(3): P30.
Brouns, F., B. Kettlitz, et al. (2002). "Resistant starch and “the butyrate revolution”." Trends in Food Science & Technology **13**(8): 251-261.
Brown, A. J., S. M. Goldsworthy, et al. (2003). "The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids." J Biol Chem **278**(13): 11312-11319.
Brown, I., S. Macnamara, et al. (2000). "The use of high amylose maize starch in the preparation of nutritional food." Food Australia **52**: 22-26.
Brown, K. H., J. A. Rivera, et al. (2004). "International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control." Food Nutr Bull **25**(1 Suppl 2): S99-203.
Brugger, D., M. Hanauer, et al. (2015). "Using piglets as an animal model: Preliminary results on the impact of short-term marginal zinc deficiency on zinc acquisition and storage dependent gene expression in jejunal and colonic tissue." Perspectives in Science **3**(1-4): 30-31.
Bruzzese, E., M. Volpicelli, et al. (2009). "A formula containing galacto- and fructo-oligosaccharides prevents intestinal and extra-intestinal infections: an observational study." Clin Nutr **28**.
Buleon, A., P. Colonna, et al. (1998). "Starch granules: structure and biosynthesis." Int J Biol

Macromol **23**(2): 85-112.
Burkholder, P. R. and I. McVeigh (1942). "Synthesis of Vitamins by Intestinal Bacteria." Proc Natl Acad Sci U S A **28**(7): 285-289.
Burn , J., D. T. Bishop , et al. (2008). "Effect of Aspirin or Resistant Starch on Colorectal Neoplasia in the Lynch Syndrome." New England Journal of Medicine **359**(24): 2567-2578.
Butterworth, P. J., F. J. Warren, et al. (2011). "Human α-amylase and starch digestion: An interesting marriage." Starch - Stärke **63**(7): 395-405.
Canny, G. O. and B. A. McCormick (2008). "Bacteria in the Intestine, Helpful Residents or Enemies from Within?" Infect Immun **76**(8): 3360-3373.
Champ, M. (2004). Resistant starch Starch in Food: Structure, Function and Applications A. Eliasson, Woodhead Publishing**:** 560-574.
Chang, J. Y., D. A. Antonopoulos, et al. (2008). "Decreased Diversity of the Fecal Microbiome in Recurrent Clostridium difficile—Associated Diarrhea." Journal of Infectious Diseases **197**(3): 435-438.
Christian, M. T., C. A. Edwards, et al. (2003). "Starch fermentation by faecal bacteria of infants, toddlers and adults: importance for energy salvage." Eur J Clin Nutr **57**(11): 1486-1491.
Cinquin, C., G. Le Blay, et al. (2004). "Immobilization of infant fecal microbiota and utilization in an in vitro colonic fermentation model." Microb Ecol **48**(1): 128-138.
Cinquin, C., G. Le Blay, et al. (2006). "New three-stage in vitro model for infant colonic fermentation with immobilized fecal microbiota." FEMS Microbiology Ecology **57**(2): 324-336.
Clarke, J. M., A. R. Bird, et al. (2007). "Excretion of starch and esterified short-chain fatty acids by ileostomy subjects after the ingestion of acylated starches." The American Journal of Clinical Nutrition **86**(4): 1146-1151.
Clarridge, J. E. (2004). "Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases." Clinical Microbiology Reviews **17**(4): 840-862.
Coles, L. T., P. J. Moughan, et al. (2005). "In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals." Animal Feed Science and Technology **123-124**: 421444.
Condomina, J., T. Zornoza-Sabina, et al. (2002). "Kinetics of zinc transport in vitro in rat small intestine and colon: interaction with copper." Eur J Pharm Sci **16**(4-5): 289-295.
Conlon, M. A. and A. R. Bird (2015). "The impact of diet and lifestyle on gut microbiota and human health." Nutrients **7**(1): 17-44.
Conlon, M. A., C. A. Kerr, et al. (2012). "Resistant Starches Protect against Colonic DNA Damage and Alter Microbiota and Gene Expression in Rats Fed a Western Diet." The journal of nutrition **142**(5): 832-840.
Cook, S. I. and J. H. Sellin (1998). "Review article: short chain fatty acids in health and disease." Aliment Pharmacol Ther **12**(6): 499-507.
Corpet, D. E. and F. Pierre (2005). "How good are rodent models of carcinogenesis in predicting efficacy in humans? A systematic review and meta-analysis of colon chemoprevention in rats, mice and men." Eur J Cancer **41**(13): 1911-1922.
Coudray, C., J. Bellanger, et al. (1997). "Effect of soluble or partly soluble dietary fibres supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men." Eur J Clin Nutr **51**(6): 375-380.
Coudray, C., C. Demigne, et al. (2003). "Effects of dietary fibers on magnesium absorption in animals and humans." J Nutr **133**(1): 1-4.
Coudray, C., C. Feillet-Coudray, et al. (2006). "Dietary Inulin Intake and Age can Affect Intestinal Absorption of Zinc and Copper in Rats." Journal of Nutrition **136**: 117-122.
Cousins, R. J. (2010). "Gastrointestinal factors influencing zinc absorption and homeostasis." Int J Vitam Nutr Res **80**(4-5): 243-248.
Cousins, R. J., J. P. Liuzzi, et al. (2006). "Mammalian zinc transport, trafficking, and signals." J Biol Chem **281**(34): 24085-24089.
Coxam, V. (2007). "Current data with inulin-type fructans and calcium, targeting bone health in

adults." J Nutr **137**(11 Suppl): 2527S-2533S.
Cragg, R. A., S. R. Phillips, et al. (2005). "Homeostatic regulation of zinc transporters in the human small intestine by dietary zinc supplementation." Gut **54**(4): 469-478.
Crane, R. K. (1960). "Intestinal absorption of sugars." Physiol Rev **40**: 789-825.
Cuello-Garcia, C. A., A. Fiocchi, et al. (2016). "World Allergy Organization-McMaster University Guidelines for Allergic Disease Prevention (GLAD-P): Prebiotics." World Allergy Organization Journal **9**(1): 1-10.
Cummings, J., E. Pomare, et al. (1987). "Short chain fatty acids in human large intestine, portal, hepatic and venous blood." Gut **28**(10): 1221-1227.
Cummings, J. H. (1981). "Short chain fatty acids in the human colon." Gut **22**(9): 763-779.
Daniel, M., E. Wisker, et al. (1997). "Fermentation in human subjects of nonstarch polysaccharides in mixed diets, but not in a barley fiber concentrate, could be predicted by in vitro fermentation using human fecal inocula." J Nutr **127**(10): 1981-1988.
David, L. A., C. F. Maurice, et al. (2014). "Diet rapidly and reproducibly alters the human gut microbiome." Nature **505**(7484): 559-563.
Davies, N. T. (1980). "Studies on the absorption of zinc by rat intestine." Br J Nutr **43**(1): 189-203.
Davis, L. M., I. Martinez, et al. (2010). "A dose dependent impact of prebiotic galactooligosaccharides on the intestinal microbiota of healthy adults." Int J Food Microbiol **144**(2): 285-292.
De Filippo, C., D. Cavalieri, et al. (2010). "Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa." Proc Natl Acad Sci U S A **107**(33): 14691-14696.
de Groot, A. P., H. P. Til, et al. (1974). "Two-year feeding amd multigeneration studies in rats on five chemically modified starches." Food Cosmet Toxicol **12**(5-6): 651-663.
De Vizia, B., F. Ciccimarra, et al. (1975). "Digestibility of starches in infants and children." J Pediatr **86**(1): 50-55.
de Vrese, M. and P. R. Marteau (2007). "Probiotics and prebiotics: effects on diarrhea." J Nutr **137**(3 Suppl 2): 803S-811S.
DeGruttola, A. K., D. Low, et al. (2016). "Current understanding of dysbiosis in disease in human and animal models." Inflamm Bowel Dis **22**(5): 1137-1150.
den Besten, G., K. van Eunen, et al. (2013). "The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism." Journal of Lipid Research **54**(9): 2325-2340.
Dhital, S., A. H.-M. Lin, et al. (2013). "Mammalian Mucosal α-Glucosidases Coordinate with α-Amylase in the Initial Starch Hydrolysis Stage to Have a Role in Starch Digestion beyond Glucogenesis." PLoS One **8**(4): e62546.
Dogra, S., O. Sakwinska, et al. (2015). "Dynamics of infant gut microbiota are influenced by delivery mode and gestational duration and are associated with subsequent adiposity." MBio **6**(1).
Donohoe, D. R., N. Garge, et al. (2011). "The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon." Cell Metab **13**(5): 517-526.
Ducros, V., J. Arnaud, et al. (2005). "Influence of short-chain fructo-oligosaccharides (sc-FOS) on absorption of Cu, Zn, and Se in healthy postmenopausal women." J Am Coll Nutr **24**(1): 30-37.
Dufner-Beattie, J., F. Wang, et al. (2003). "The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice." J Biol Chem **278**(35): 33474-33481.
Duggan, C., M. E. Penny, et al. (2003). "Oligofructose-supplemented infant cereal: 2 randomized, blinded, community-based trials in Peruvian infants." Am J Clin Nutr **77**(4): 937-942.
Duranti, S., F. Turroni, et al. (2014). "Genomic characterization and transcriptional studies of the starch-utilizing strain Bifidobacterium adolescentis 22L." Appl Environ Microbiol **80**(19): 6080-6090.
Eaton, S. B. (2006). "The ancestral human diet: what was it and should it be a paradigm for contemporary nutrition?" Proc Nutr Soc **65**(1): 1-6.
Eckburg, P. B., E. M. Bik, et al. (2005). "Diversity of the human intestinal microbial flora." Science **308**(5728): 1635-1638.

Edgar, R. C. (2010). "Search and clustering orders of magnitude faster than BLAST." Bioinformatics.
Edwards, C. A., G. Gibson, et al. (1996). "In Vitro Method for Quantification of the Fermentation of Starch by Human Faecal Bacteria." journal of the science of food and agriculture **71**(2): 209-217.
Edwards, C. A. and A. M. Parrett (2003). "Dietary fibre in infancy and childhood." Proceedings of the Nutrition Society **62**(01): 17-23.
El Aidy, S., T. G. Dinan, et al. (2015). "Gut Microbiota: The Conductor in the Orchestra of Immune–Neuroendocrine Communication." Clinical Therapeutics **37**(5): 954-967.
Endo, A., M. L. Tang, et al. (2015). "1.8 Gut microbiota in infants." World Rev Nutr Diet **113**: 87-91.
Englyst, H., H. S. Wiggins, et al. (1982). "Determination of the non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates." Analyst **107**(1272): 307-318.
Englyst, H. N., S. M. Kingman, et al. (1992). "Classification and measurement of nutritionally important starch fractions." European Journal of Clinical Nutrition **46**: S33-S50.
Englyst, H. N., H. Trowell, et al. (1987). "Dietary fiber and resistant starch." Am J Clin Nutr **46**(6): 873-874.
Faith, J. J., J. L. Guruge, et al. (2013). "The long-term stability of the human gut microbiota." Science **341**(6141): 1237439.
Faith, J. J., N. P. McNulty, et al. (2011). "Predicting a human gut microbiota's response to diet in gnotobiotic mice." Science **333**(6038): 101-104.
Fallani, M., S. Amarri, et al. (2011). "Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres." Microbiology **157**(Pt 5): 1385-1392.
Fanaro, S., B. Marten, et al. (2009). "Galacto-oligosaccharides are bifidogenic and safe at weaning: a double-blind randomized multicenter study." J Pediatr Gastroenterol Nutr **48**(1): 82-88.
FASEB (1979). "Evaluation of the Health Aspects of Starch and Modified Starches as Food Ingredients." Federation of American Societies for Experimental Biology (FASEB) for Washington (DC): Food and Drug Administration, U.S. (FDA), Bureau of Foods. .
FCC (2014). Food starch, modified. In: Food Chemicals Codex, 9th edition. Rockville (MD): United States Pharmacopeial Convention (USP), pp. 496-499. .
Fewtrell, M. S., J. B. Morgan, et al. (2007). "Optimal duration of exclusive breastfeeding: what is the evidence to support current recommendations?" The American Journal of Clinical Nutrition **85**(2): 635S-638S.
Field, M. (2003). "Intestinal ion transport and the pathophysiology of diarrhea." Journal of Clinical Investigation **111**(7): 931-943.
Field, M., D. Fromm, et al. (1972). "Effect of cholera enterotoxin on ion transport across isolated ileal mucosa." J Clin Invest **51**(4): 796-804.
Figueroa-Gonzalez, I., G. Quijano, et al. (2011). "Probiotics and prebiotics--perspectives and challenges." J Sci Food Agric **91**(8): 1341-1348.
Filer, L. J. (1971). "Modified Food Starches for Use in Infant Foods." Nutrition Reviews **29**(3): 55-59.
Firmansyah, A., P. G. Dwipoerwantoro, et al. (2011). "Improved growth of toddlers fed a milk containing synbiotics." Asia Pac J Clin Nutr **20**(1): 69-76.
Fischer Walker, C. L., M. Ezzati, et al. (2009). "Global and regional child mortality and burden of disease attributable to zinc deficiency." Eur J Clin Nutr **63**(5): 591-597.
Flamm, G., W. Glinsmann, et al. (2001). "Inulin and oligofructose as dietary fiber: a review of the evidence." Crit Rev Food Sci Nutr **41**(5): 353-362.
Flint, H. J., K. P. Scott, et al. (2012). "Microbial degradation of complex carbohydrates in the gut." Gut Microbes **3**(4): 289-306.
Fouhse, J. M., M. G. Gänzle, et al. (2015). "High Amylose Starch with Low In Vitro Digestibility Stimulates Hindgut Fermentation and Has a Bifidogenic Effect in Weaned Pigs." The

journal of nutrition **145**(11): 2464-2470.
Freedman, S. B., D. Pasichnyk, et al. (2015). "Gastroenteritis Therapies in Developed Countries: Systematic Review and Meta-Analysis." PLoS One **10**(6): e0128754.
Fritz, J. V., M. S. Desai, et al. (2013). "From meta-omics to causality: experimental models for human microbiome research." Microbiome **1**: 14-14.
Fuentes-Zaragoza, E., M. J. Riquelme-Navarrete, et al. (2010). "Resistant starch as functional ingredient: A review." food research international **43**(4): 931-942.
Fuentes-Zaragoza, E., E. Sanchez-Zapata, et al. (2011). "Resistant starch as prebiotic: A review." Starch-Starke **63**(7): 406-415.
Fukuda, S., H. Toh, et al. (2011). "Bifidobacteria can protect from enteropathogenic infection through production of acetate." Nature **469**(7331): 543-547.
Gallagher, S. R. and P. R. Desjardins (2001). Quantitation of DNA and RNA with Absorption and Fluorescence Spectroscopy. Current Protocols in Human Genetics, John Wiley & Sons, Inc.
Gareau, M. G., E. Wine, et al. (2010). "Probiotics prevent death caused by Citrobacter rodentium infection in neonatal mice." J Infect Dis **201**(1): 81-91.
Gee, J., R. Faulks, et al. (1991). "Physiological effects of retrograded, alpha-amylase-resistant cornstarch in rats." The Journal of Nutrition **121**(1): 44-49.
George, M., K. E. Nord, et al. (1996). "Faecal microflora and urease activity during the first six months of infancy." Ups J Med Sci **101**(3): 233-250.
Gerritsen, J., H. Smidt, et al. (2011). "Intestinal microbiota in human health and disease: the impact of probiotics." Genes & Nutrition **6**(3): 209-240.
Gibson, G. R., H. M. Probert, et al. (2004). "Dietary modulation of the human colonic microbiota: updating the concept of prebiotics." Nutr Res Rev **17**(2): 259-275.
Gibson, G. R. and M. B. Roberfroid (1995). "Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics." J Nutr **125**(6): 1401-1412.
Gibson, M. K., T. S. Crofts, et al. (2015). "Antibiotics and the developing infant gut microbiota and resistome." Curr Opin Microbiol **27**: 51-56.
Gielda, L. M. and V. J. DiRita (2012). "Zinc competition among the intestinal microbiota." MBio **3**(4): e00171-00112.
Gijs, d. B., K. Van Eunen, et al. (2013). "The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism." Journal of Lipid Research **54**(9): 2325-2340.
Gisbert-Gonzalez, S. L. and F. Torres-Molina (1996). "Zinc uptake in five sectors of the rat gastrointestinal tract: kinetic study in the whole colon." Pharm Res **13**(8): 1154-1161.
Goñi, I. and N. Martin-Carrón (1998). "In vitro fermentation and hydration properties of commercial dietary fiber-rich supplements." Nutrition Research **18**(6): 1077-1089.
Govers, M. J. A. P., N. J. Gannon, et al. (1999). "Wheat bran affects the site of fermentation of resistant starch and luminal indexes related to colon cancer risk: a study in pigs." Gut **45**(6): 840-847.
Gower, B. A., R. Bergman, et al. (2016). "Baseline insulin sensitivity affects response to high-amylose maize resistant starch in women: a randomized, controlled trial." Nutr Metab (Lond) **13**(1): 2.
Grice, E. A. and J. A. Segre (2012). "The human microbiome: our second genome." Annu Rev Genomics Hum Genet **13**: 151-170.
Gronlund, M. M., O. P. Lehtonen, et al. (1999). "Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery." J Pediatr Gastroenterol Nutr **28**(1): 19-25.
Guaraldi, F. and G. Salvatori (2012). "Effect of breast and formula feeding on gut microbiota shaping in newborns." Front Cell Infect Microbiol.
Hahn, S., Y. Kim, et al. (2001). "Reduced osmolarity oral rehydration solution for treating dehydration due to diarrhoea in children: systematic review." BMJ **323**(7304): 81-85.
Hallam, M. C., D. Barile, et al. (2014). "Maternal high-protein or high-prebiotic-fiber diets affect maternal milk composition and gut microbiota in rat dams and their offspring." Obesity (Silver Spring) **22**(11): 2344-2351.

Hambidge, K., L. Miller, et al. (2010). "Zinc bioavailability and homeostasis." Am J Clin Nutr **91**(5): 1478S-1483S.
Han, F., M. Liu, et al. (2012). "Synthesis, characterization and functional properties of low substituted acetylated corn starch." International Journal of Biological Macromolecules **50**(4): 1026-1034.
Hantke, K. (2001). Bacterial zinc transporters and regulators. Zinc Biochemistry, Physiology, and Homeostasis: Recent Insights and Current Trends. W. Maret. Dordrecht, Springer Netherlands**:** 53-63.
Hara, H., A. Konishi, et al. (2000). "Contribution of the cecum and colon to zinc absorption in rats." J Nutr **130**(1): 83-89.
Hardy, H., J. Harris, et al. (2013). "Probiotics, Prebiotics and Immunomodulation of Gut Mucosal Defences: Homeostasis and Immunopathology." Nutrients **5**(6): 1869-1912.
Harri, M. and N. Päivi (2006). In Vitro Methods to Model the Gastrointestinal Tract. Gastrointestinal Microbiology, CRC Press**:** 237-252.
Hasjim, J., Y. Ai, et al. (2013). Novel Applications of Amylose-Lipid Complex as Resistant Starch Type 5. Resistant Starch, John Wiley and Sons Ltd**:** 79-94.
Hayashi, K., H. Hara, et al. (2001). "Ingestion of insoluble dietary fibre increased zinc and iron absorption and restored growth rate and zinc absorption suppressed by dietary phytate in rats." Br J Nutr **86**(4): 443-451.
Heilig, H. G., E. G. Zoetendal, et al. (2002). "Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA." Appl Environ Microbiol **68**(1): 114-123.
Heinritz, S. N., R. Mosenthin, et al. (2013). "Use of pigs as a potential model for research into dietary modulation of the human gut microbiota." Nutr Res Rev **26**(2): 191-209.
Hiergeist, A., J. Gläsner, et al. (2015). "Analyses of Intestinal Microbiota: Culture versus Sequencing." ILAR Journal **56**(2): 228-240.
Hogenkamp, A., L. M. Knippels, et al. (2015). "Supplementation of Mice with Specific Nondigestible Oligosaccharides during Pregnancy or Lactation Leads to Diminished Sensitization and Allergy in the Female Offspring." J Nutr **145**(5): 996-1002.
Hotz, C. and K. H. Brown (2004). "Assessment of the risk of zinc deficiency in populations and options for its control. International Zinc Consultative Group (iZinCG) Technical Document #1." Food Nutr Bull **25**: S94-S204.
Hsieh, C.-Y., T. Osaka, et al. (2015). "Strengthening of the intestinal epithelial tight junction by Bifidobacterium bifidum." Physiol Rep **3**(3): e12327.
Hunt, J. B., E. J. Elliott, et al. (1992). "Water and solute absorption from hypotonic glucose-electrolyte solutions in human jejunum." Gut **33**(4): 479-483.
Hunt, J. R. (2003). "Bioavailability of iron, zinc, and other trace minerals from vegetarian diets." Am J Clin Nutr **78**(3 Suppl): 633S-639S.
Hylander, E., K. Ladefoged, et al. (1990). "Calcium absorption after intestinal resection. The importance of a preserved colon." Scand J Gastroenterol **25**(7): 705-710.
Ichimura, A., S. Hasegawa, et al. (2014). "Free fatty acid receptors as therapeutic targets for the treatment of diabetes." Frontiers in Pharmacology **5**: 236.
Ismail, I. H., P. V. Licciardi, et al. (2013). "Probiotic effects in allergic disease." Journal of Paediatrics and Child Health **49**(9): 709-715.
Jandhyala, S. M., R. Talukdar, et al. (2015). "Role of the normal gut microbiota." World J Gastroenterol **21**(29): 8787-8803.
JEFCA (1982a). Starch acetate. In: Toxicological Evaluation on Certain Veterinary Drug

Residues in Food. 26th Meeting of the Joint FAO/WHO Expert Committee on Food

Additives (JECFA). (WHO Food Additive Series No. 17). Geneva, Switz.: World Health

Organization (WHO) Available at: http://www.inchem.org/documents/jecfa/jecmono/v17je20.htm. .
Jeong, J. and D. J. Eide (2013). "The SLC39 family of zinc transporters." Molecular Aspects of Medicine **34**(2–3): 612-619.
Jiang, X., B. Li, et al. (2013). "Shifts in Bacterial Community Compositions during in vtro

fermentation of amylopectin and resistant starch by colonic onocula of Pigs." Journal of Food and Nutrition Research **1**(6): 156-163.
Jobling, S. (2004). "Improving starch for food and industrial applications." Current opinion in plant biology **7**(2): 210-218.
Jobling, S. (2004). "Improving starch for food and industrial applications." Curr Opin Plant Biol **7**(2): 210-218.
John, E., T. C. Laskow, et al. (2010). "Zinc in innate and adaptive tumor immunity." Journal of Translational Medicine **8**: 118-118.
Johnson, J. L., M. B. Jones, et al. (2015). "Polysaccharide A from the capsule of Bacteroides fragilis induces clonal CD4+ T cell expansion." J Biol Chem **290**(8): 5007-5014.
Jonkers, D. M. A. E. (2016). "Microbial perturbations and modulation in conditions associated with malnutrition and malabsorption." Best Practice & Research Clinical Gastroenterology **30**(2): 161-172.
Kailasapathy, K. and J. Chin (2000). "Survival and therapeutic potential of probiotic organisms with reference to Lactobacillus acidophilus and Bifidobacterium spp." Immunol Cell Biol **78**(1): 80-88.
Kajosaari, M. (1994). "Atopy prevention in childhood: the role of diet. Prospective 5-year follow-up of high-risk infants with six months exclusive breastfeeding and solid food elimination." Pediatr Allergy Immunol **5**(6 Suppl): 26-28.
Kaoutari, A. E., F. Armougom, et al. (2013). "The abundance and variety of carbohydrate-active enzymes in the human gut microbiota." Nat Rev Micro **11**(7): 497-504.
Keely, S. J., M. H. Montrose, et al. (2009). Electrolyte Secretion and Absorption: Small Intestine and Colon. Textbook of Gastroenterology, Blackwell Publishing Ltd.**:** 330-367.
Keenan, M. J., J. Zhou, et al. (2006). "Effects of Resistant Starch, A Non-digestible Fermentable Fiber, on Reducing Body Fat." Obesity **14**(9): 1523-1534.
Khoruts, A. and M. J. Sadowsky (2016). "Understanding the mechanisms of faecal microbiota transplantation." Nat Rev Gastroenterol Hepatol **advance online publication**.
Kim, B. S., J. N. Kim, et al. (2011). "In vitro culture conditions for maintaining a complex population of human gastrointestinal tract microbiota." J Biomed Biotechnol **2011**: 838040.
King, J. C. (2010). "Does zinc absorption reflect zinc status?" Int J Vitam Nutr Res **80**(4-5): 300-306.
Kleessen, B., G. Stoof, et al. (1997). "Feeding resistant starch affects fecal and cecal microflora and short-chain fatty acids in rats." J Anim Sci **75**(9): 2453-2462.
Koenig, J. E., A. Spor, et al. (2011). "Succession of microbial consortia in the developing infant gut microbiome." Proc Natl Acad Sci U S A **108**.
Korpela, K., A. Salonen, et al. (2016). "Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children." Nat Commun **7**: 10410.
Kotloff, K., J. Nataro, et al. (2013). "Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study." Lancet **382**(9888): 209-222.
Kramer, M. S. and R. Kakuma (2012). "Optimal duration of exclusive breastfeeding." Cochrane Database Syst Rev **8**: CD003517.
Krebs, N. F. (2000). "Overview of zinc absorption and excretion in the human gastrointestinal tract." J Nutr **130**(5S Suppl): 1374S-1377S.
Krebs, N. F. (2013). "Update on zinc deficiency and excess in clinical pediatric practice." Ann Nutr Metab **62** (Suppl 1): 19-29.
Krebs, N. F., L. V. Miller, et al. (2014). "Zinc deficiency in infants and children: a review of its complex and synergistic interactions." Paediatr Int Child Health **34**(4): 279-288.
Krishnan, S., V. M. Rajendran, et al. (2003). "Apical NHE isoforms differentially regulate butyrate-stimulated Na absorption in rat distal colon." Am J Physiol Cell Physiol **285**(5): C1246-1254.
Krishnan, S., B. S. Ramakrishna, et al. (1999). "Stimulation of sodium chloride absorption from secreting rat colon by short-chain fatty acids." Dig Dis Sci **44**(9): 1924-1930.
Kumar, V., A. Sinha, et al. (2010). "Dietary roles of phytate and phytase in human nutrtion: A review." Food Chemistry **120**: 945-959.

Kunzelmann, K. and M. Mall (2002). "Electrolyte transport in the mammalian colon: mechanisms and implications for disease." Physiol Rev **82**(1): 245-289.
Lanciers, S., D. I. Mehta, et al. (1998). "Modified food starches in baby foods." Indian J Pediatr **65**(4): 541-546.
Lawrence, D. A., W. Ana, et al. (2015). "Gut Microbial Succession Follows Acute Secretory Diarrhea in Humans." MBio **6**(3): e00381-00315.
Layden, B. T., A. R. Angueira, et al. (2013). "Short chain fatty acids and their receptors: new metabolic targets." Translational Research **161**(3): 131-140.
Lazzerini, M. (2016). "Oral zinc provision in acute diarrhea." Current Opinion in Clinical Nutrition & Metabolic Care **19**(3): 239-243.
Le Leu, R. K., I. L. Brown, et al. (2007). "Effect of dietary resistant starch and protein on colonic fermentation and intestinal tumourigenesis in rats." Carcinogenesis **28**(2): 240-245.
Le Poul, E., C. Loison, et al. (2003). "Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation." J Biol Chem **278**(28): 25481-25489.
Lebenthal, E. and P. C. Lee (1980). "Development of functional responses in human exocrine pancreas." Pediatrics **66**(4): 556-560.
Lee, H. H., A. S. Prasad, et al. (1989). "Zinc absorption in human small intestine." Am J Physiol **256**(1 Pt 1): G87-91.
Lee, P. C., S. Werlin, et al. (2004). "Glucoamylase activity in infants and children: normal values and relationship to symptoms and histological findings." J Pediatr Gastroenterol Nutr **39**(2): 161-165.
Lee, Y.-K. (2013). "Effects of Diet on Gut Microbiota Profile and the Implications for Health and Disease." Bioscience of Microbiota, Food and Health **32**(1): 1-12.
Leitch, E., A. Walker, et al. (2007). "Selective colonization of insoluble substrates by human faecal bacteria." Environmental Microbiology **9**(3): 667-669.
Ley, R. E., D. A. Peterson, et al. (2006). "Ecological and evolutionary forces shaping microbial diversity in the human intestine." Cell **124**(4): 837-848.
Lim, Y.-M., M. B. Barnes, et al. (2014). "Esterification of high amylose starch with short chain fatty acids modulates degradation by Bifidobacterium spp." Journal of Functional Foods **6**: 137-146.
Lindenmayer, G. W., R. J. Stoltzfus, et al. (2014). "Interactions between Zinc Deficiency and Environmental Enteropathy in Developing Countries." Adv Nutr **5**(1): 1-6.
Liu, S., F. Ren, et al. (2015). "Starch and starch hydrolysates are favorable carbon sources for Bifidobacteria in the human gut." BMC Microbiology **15**(1).
Long, W., Z. Xue, et al. (2015). "Differential responses of gut microbiota to the same prebiotic formula in oligotrophic and eutrophic batch fermentation systems." Sci Rep **5**: 13469.
Lonnerdal, B. (2000). "Dietary factors influencing zinc absorption." J Nutr **130**(5S Suppl): 1378S-1383S.
Lopez, H. W., C. Coudray, et al. (1998). "Intestinal fermentation lessens the inhibitory effects of phytic acid on mineral utilization in rats." J Nutr **128**(7): 1192-1198.
Lopez, H. W., F. Leenhardt, et al. (2002). "Minerals and phytic acid interactions: is it a real problem for human nutrition?" International Journal of Food Science & Technology **37**(7): 727-739.
Lowe, N. M., K. Fekete, et al. (2009). "Methods of assessment of zinc status in humans: a systematic review." Am J Clin Nutr **89**(6): 2040S-2051S.
Lozupone, C., J. Stombaugh, et al. (2012). "Diversity, stability and resilience of the human gut microbiota." Nature **489**.
Lupton, J. R., V. A. Betteridge, et al. (2009). "Codex final definition of dietary fibre: issues of implementation." Quality Assurance and Safety of Crops & Foods **1**(4): 206-212.
Lyte, M., A. Chapel, et al. (2016). "Resistant Starch Alters the Microbiota-Gut Brain Axis: Implications for Dietary Modulation of Behavior." PLoS One **11**(1): e0146406.
Macfarlane, G. T. and H. N. Englyst (1986). "Starch utilization by the human large intestinal microflora." J Appl Bacteriol **60**(3): 195-201.
Macfarlane, S., G. T. Macfarlane, et al. (2006). "Review article: prebiotics in the gastrointestinal tract." Aliment Pharmacol Ther **24**(5): 701-714.

Mahalanabis, D., A. B. Choudhuri, et al. (1973). "Oral fluid therapy of cholera among Bangladesh refugees." Johns Hopkins Med J **132**(4): 197-205.
Mahalanabis, D., A. S. Faruque, et al. (1995). "Hypotonic oral rehydration solution in acute diarrhoea: a controlled clinical trial." Acta Paediatr **84**(3): 289-293.
Maki, K. C., C. L. Pelkman, et al. (2012). "Resistant Starch from High-Amylose Maize Increases Insulin Sensitivity in Overweight and Obese Men." The journal of nutrition **142**(4): 717-723.
Manary, M., S. Abrams, et al. (2010). "Perturbed Zinc Homeostasis in Rural 3–5-y-Old Malawian Children Is Associated With Abnormalities in Intestinal Permeability Attributed to Tropical Enteropathy." Pediatr Res **67**: 671-675.
Maret, W. and A. Krezel (2007). "Cellular zinc and redox buffering capacity of metallothionein/thionein in health and disease." Mol Med **13**(7-8): 371-375.
Martinez, I., J. Kim, et al. (2010). "Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects." PLoS One **5**(11): e15046.
Maslowski, K. M., A. T. Vieira, et al. (2009). "Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43." Nature **461**(7268): 1282-1286.
Matamoros, S., C. Gras-Leguen, et al. "Development of intestinal microbiota in infants and its impact on health." Trends in microbiology **21**(4): 167-173.
Mathers, J. C. and T. M. Wolever (2009). Digestion and Metabolism of Carbohydrates. Introduction to Human Nutrition. M. Gibney, S. Lanham-New, A. Cassidy and H. Vorster, Wiley-Blackwell.
Matsuki, T., K. Watanabe, et al. (1999). "Distribution of Bifidobacterial Species in Human Intestinal Microflora Examined with 16S rRNA-Gene-Targeted Species-Specific Primers." Applied and environmental microbiology **65**(10): 4506-4512.
May, T., C. Westcott, et al. (2015). "Resistant starch does not affect zinc homeostasis in rural Malawian children." J Trace Elem Med Biol **30**: 43-48.
Mazmanian, S. K., C. H. Liu, et al. (2005). "An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system." Cell **122**(1): 107-118.
McInnes, P. and M. Cutting (2010). "Manual of Procedures

for Human Microbiome Project:." http://hmpdacc.org/resources/tools_protocols.php.
McIntyre, A., P. R. Gibson, et al. (1993). "Butyrate production from dietary fibre and protection against large bowel cancer in a rat model." Gut **34**(3): 386-391.
McKain, N., B. Genc, et al. (2013). "Differential recovery of bacterial and archaeal 16S rRNA genes from ruminal digesta in response to glycerol as cryoprotectant." Journal of Microbiological Methods **95**(3): 381-383.
McOrist, A. L., G. C. Abell, et al. (2008). "Bacterial population dynamics and faecal short-chain fatty acid (SCFA) concentrations in healthy humans." Br J Nutr **100**(1): 138-146.
McWhirter, K. and C. Dunn (1986). "Development of high amylose maize production in Australia." Agronomy Society of New Zealand Special Publication No. 5: 86-91.
Medina, V., B. Edmonds, et al. (1997). "Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway." Cancer Res **57**(17): 3697-3707.
Milani, C., G. A. Lugli, et al. (2015). "Bifidobacteria exhibit social behavior through carbohydrate resource sharing in the gut." Sci Rep **5**: 15782.
Miller, L. V., K. M. Hambidge, et al. (2015). "Zinc Absorption Is Not Related to Dietary Phytate Intake in Infants and Young Children Based on Modeling Combined Data from Multiple Studies." J Nutr **145**(8): 1763-1769.
Miyazaki, M., P. Van Hung, et al. (2006). "Recent advances in application of modified starches for breadmaking." Trends in Food Science & Technology **17**(11): 591-599.
Molly, K., M. Vande Woestyne, et al. (1993). "Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem." Applied microbiology and biotechnology **39**(2): 254-258.
Monira, S., S. Nakamura, et al. (2011). "Gut microbiota of healthy and malnourished children in bangladesh." Front Microbiol **2**: 228.
Moore, N., C. Chao, et al. (2003). "Effects of fructo-oligosaccharide-supplemented infant cereal: a

double-blind, randomized trial." Br J Nutr **90**(3): 581-587.
Morita, T., S. Kasaoka, et al. (2005). "Comparative Effects of Acetylated and Unmodified High-amylose Maize Starch in Rats." Starch - Stärke **57**(6): 246-253.
Mowat, A. M. and W. W. Agace (2014). "Regional specialization within the intestinal immune system." Nat Rev Immunol **14**(10): 667-685.
Moya, A. and M. Ferrer (2016). "Functional Redundancy-Induced Stability of Gut Microbiota Subjected to Disturbance." Trends in microbiology **24**(5): 402-413.
Mueller, N. T., E. Bakacs, et al. (2015). "The infant microbiome development: mom matters." Trends in Molecular Medicine **21**(2): 109-117.
Mugambi, M. N., A. Musekiwa, et al. (2012). "Synbiotics, probiotics or prebiotics in infant formula for full term infants: a systematic review." Nutrition Journal **11**(1): 1-32.
Muir, J. G., A. Birkett, et al. (1995). "Food processing and maize variety affects amounts of starch escaping digestion in the small intestine." Am J Clin Nutr **61**(1): 82-89.
Muraro, A., S. Halken, et al. (2014). "EAACI food allergy and anaphylaxis guidelines. Primary prevention of food allergy." Allergy **69**(5): 590-601.
Murray, J.-A. M. D., P. McMullin, et al. (2012). "The effect of freezing on the fermentative activity of equine faecal inocula for use in an in vitro gas production technique." Animal Feed Science and Technology **178**(3–4): 175-182.
Nadkarni, M. A., F. E. Martin, et al. (2002). "Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set." Microbiology **148**(Pt 1): 257-266.
Nassos, P. S., A. D. King, et al. (1988). "Lactic acid concentration as an indicator of acceptability in refrigerated or freeze-thawed ground beef." Applied and environmental microbiology **54**(3): 822-823.
Nauta, A. J., K. Ben Amor, et al. (2013). "Relevance of pre- and postnatal nutrition to development and interplay between the microbiota and metabolic and immune systems." Am J Clin Nutr **98**(2): 586S-593S.
NHMRC (2013) "Infant Feeding Guidelines." https://www.eatforhealth.gov.au/sites/default/files/files/the_guidelines/n56b_infant_feeding_summary_130808.pdf.
Niittynen, L., K. Kajander, et al. (2007). "Galacto-oligosaccharides and bowel function." Scandinavian Journal of Food & Nutrition **51**(2): 62-66.
Noguera, T., R. Wotring, et al. (2014). "Resolution of acute gastroenteritis symptoms in children and adults treated with a novel polyphenol-based prebiotic." World J Gastroenterol **20**(34): 12301-12307.
Nordgaard, I., P. B. Mortensen, et al. (1995). "Small intestinal malabsorption and colonic fermentation of resistant starch and resistant peptides to short-chain fatty acids." Nutrition **11**(2): 129-137.
O'Donnell, M. D. and N. J. Miller (1980). "Plasma pancreatic and salivary-type amylase and immunoreactive trypsin concentrations: variations with age and reference ranges for children." Clin Chim Acta **104**(3): 265-273.
Odamaki, T., A. Horigome, et al. (2015). "Comparative Genomics Revealed Genetic Diversity and Species/Strain-Level Differences in Carbohydrate Metabolism of Three Probiotic Bifidobacterial Species." International Journal of Genomics **2015**: 12.
Ogawa, K., R. A. Ben, et al. (1992). "Volatile fatty acids, lactic acid, and pH in the stools of breast-fed and bottle-fed infants." J Pediatr Gastroenterol Nutr **15**(3): 248-252.
Okada, H., C. Kuhn, et al. (2010). "The 'hygiene hypothesis' for autoimmune and allergic diseases: an update." Clin Exp Immunol **160**(1): 1-9.
Olesen, M., J. J. Rumessen, et al. (1994). "Intestinal transport and fermentation of resistant starch evaluated by the hydrogen breath test." Eur J Clin Nutr **48**(10): 692-701.
Olszak, T., D. An, et al. (2012). "Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function." Science (New York, N.Y.) **336**(6080): 489-493.
Ordiz, M. I., T. D. May, et al. (2015). "The effect of dietary resistant starch type 2 on the microbiota and markers of gut inflammation in rural Malawi children." Microbiome **3**: 37.
Orel, R. and L. V. Reberšak (2016). "Prebiotics Use in Children." 181-193.
Outten, C. E. and T. V. O'Halloran (2001). "Femtomolar sensitivity of metalloregulatory proteins

controlling zinc homeostasis." Science **292**(5526): 2488-2492.
Ouwehand, A., E. Isolauri, et al. (2002). "The role of the intestinal microflora for the development of the immune system in early childhood." Eur J Nutr **41 Suppl 1**: I32-37.
Paeschke, T. M. and W. R. Aimutis (2010). Introduction to Fiber and Nondigestible Carbohydrates: Definition, Health Aspects, and Perspectives. Nondigestible Carbohydrates and Digestive Health, Wiley-Blackwell**:** 1-13.
Palmer, C., E. M. Bik, et al. (2007). "Development of the human infant intestinal microbiota." PLoS Biol **5**(7): e177.
Parrett, A. and C. Edwards (1997). "In vitro fermentation of carbohydrate by breast fed and formula fed infants." Archives of Disease in Childhood **76**: 249-253.
Parrett, A. M., C. A. Edwards, et al. (1997). "Colonic fermentation capacity in vitro: development during weaning in breast-fed infants is slower for complex carbohydrates than for sugars." The American journal of clinical nutrition **65**(4): 927-933.
Pastorelli, L., C. De Salvo, et al. (2013). "Central Role of the Gut Epithelial Barrier in the Pathogenesis of Chronic Intestinal Inflammation: Lessons Learned from Animal Models and Human Genetics." Frontiers in Immunology **4**: 280.
Payne, A. N., A. Zihler, et al. (2012). "Advances and perspectives in in vitro human gut fermentation modeling." Trends in Biotechnology **30**(1): 17-25.
Peng, L., Z. He, et al. (2007). "Effects of butyrate on intestinal barrier function in a Caco-2 cell monolayer model of intestinal barrier." Pediatr Res **61**(1): 37-41.
Pfaffl, M. W. and W. Windisch (2003). "Influence of zinc deficiency on the mRNA expression

of zinc transporters in adult rats." J. Trace Elem. Med. BioL **17**: 97-106.
Picard, C., J. Fioramonti, et al. (2005). "Review article: bifidobacteria as probiotic agents – physiological effects and clinical benefits." Aliment Pharmacol Ther **22**(6): 495-512.
Piemontese, P., M. L. Gianni, et al. (2011). "Tolerance and safety evaluation in a large cohort of healthy infants fed an innovative prebiotic formula: a randomized controlled trial." PLoS One **6**(11): e28010.
Pieper, R., W. Vahjen, et al. (2012). "Dose-dependent effects of dietary zinc oxide on bacterial communities and metabolic profiles in the ileum of weaned pigs." J Anim Physiol Anim Nutr (Berl) **96**(5): 825-833.
Pokusaeva, K., G. F. Fitzgerald, et al. (2011). "Carbohydrate metabolism in Bifidobacteria." Genes and Nutrition **6**(3): 285-306.
Pompei, A., L. Cordisco, et al. (2007). "Administration of folate-producing bifidobacteria enhances folate status in Wistar rats." J Nutr **137**(12): 2742-2746.
Popovich, D. G., D. J. Jenkins, et al. (1997). "The western lowland gorilla diet has implications for the health of humans and other hominoids." J Nutr **127**(10): 2000-2005.
Pouteau, E., I. Meirim, et al. (2001). "Acetate, propionate and butyrate in plasma: determination of the concentration and isotopic enrichment by gas chromatography/mass spectrometry with positive chemical ionization." Journal of Mass Spectrometry **36**(7): 798-805.
Rabbani, G. H., T. Teka, et al. (2001). "Clinical studies in persistent diarrhea: Dietary management with green banana or pectin in Bangladeshi children." Gastroenterology **121**(3): 554-560.
Raghupathy, P., B. S. Ramakrishna, et al. (2006). "Amylase-resistant starch as adjunct to oral rehydration therapy in children with diarrhea." J Pediatr Gastroenterol Nutr **42**.
Rakoff-Nahoum, S., Y. Kong, et al. (2015). "Analysis of gene–environment interactions in postnatal development of the mammalian intestine." Proceedings of the National Academy of Sciences of the United States of America **112**(7): 1929-1936.
Ramakrishna, B. S., V. Subramanian, et al. (2008). "A randomized controlled trial of glucose versus amylase resistant starch hypo-osmolar oral rehydration solution for adult acute dehydrating diarrhea." PLoS One **3**(2): e1587.
Ramakrishna, B. S., S. Venkataraman, et al. (2006). "Tropical malabsorption." Postgrad Med J **82**(974): 779-787.
Ramakrishna, B. S., S. Venkataraman, et al. (2000). "Amylase-Resistant Starch plus Oral Rehydration Solution for Cholera." New England Journal of Medicine **342**(5): 308313.
Rao, D. E., K. V. Rao, et al. (2009). "Molecular characterization, physicochemical properties, known and potential applications of phytases: An overview." Crit Rev Biotechnol **29**(2): 182-

198.
Rao, S., R. Srinivasjois, et al. (2009). "Prebiotic supplementation in full-term neonates: A systematic review of randomized controlled trials." Archives of Pediatrics & Adolescent Medicine **163**(8): 755-764.
Rautava, S., R. Luoto, et al. (2012). "Microbial contact during pregnancy, intestinal colonization and human disease." Nat Rev Gastroenterol Hepatol **9**(10): 565-576.
Reed, S., H. Neuman, et al. (2015). "Chronic Zinc Deficiency Alters Chick Gut Microbiota Composition and Function." Nutrients **7**(12): 9768-9784.
Reed, S., H. Neuman, et al. (2015). "Chronic Zinc Deficiency Alters Chick Gut Microbiota Composition and Function." Nutrients **7**(12): 9768-9784.
Rigo-Adrover, M., S. Saldaña-Ruíz, et al. (2016). "A combination of scGOS/lcFOS with Bifidobacterium breve M-16V protects suckling rats from rotavirus gastroenteritis." European Journal of Nutrition: 1-14.
Ringel-Kulka, T., J. Cheng, et al. (2013). "Intestinal Microbiota in Healthy U.S. Young Children and Adults—A High Throughput Microarray Analysis." PLoS One **8**(5): e64315.
Rinttila, T., A. Kassinen, et al. (2004). "Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR." J Appl Microbiol **97**(6): 1166-1177.
Rios-Covian, D., P. Ruas-Madiedo, et al. (2016). "Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health." Front Microbiol **7**: 185.
Rivière, A., M. Selak, et al. (2016). "Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut." Front Microbiol **7**: 979.
Robertson, M. D., A. S. Bickerton, et al. (2005). "Insulin-sensitizing effects of dietary resistant starch and effects on skeletal muscle and adipose tissue metabolism." Am J Clin Nutr **82**(3): 559-567.
Rodriguez, B., G. Prioult, et al. (2012). "Infant gut microbiota is protective against cow's milk allergy in mice despite immature ileal T-cell response." FEMS Microbiol Ecol **79**(1): 192-202.
Roduit, C., S. Scholtens, et al. (2009). "Asthma at 8 years of age in children born by caesarean section." Thorax **64**(2): 107-113.
Roediger, W. E. (1982). "Utilization of nutrient by isolated epithelial cells of the rat colon." Gastroenterology **83**.
Rose, D. J., A. Keshavarzian, et al. (2009). "Starch-entrapped microspheres extend in vitro fecal fermentation, increase butyrate production, and influence microbiota pattern." Mol Nutr Food Res **53 Suppl 1**: S121-130.
Rose, D. J., K. Venema, et al. (2010). "Starch-entrapped microspheres show a beneficial fermentation profile and decrease in potentially harmful bacteria during in vitro fermentation in faecal microbiota obtained from patients with inflammatory bowel disease." Br J Nutr **103**(10): 1514-1524.
Rosenblum, J. L., C. L. Irwin, et al. (1988). "Starch and glucose oligosaccharides protect salivary-type amylase activity at acid pH." Am J Physiol **254**(5 Pt 1): G775-780.
Royall, D., T. M. Wolever, et al. (1990). "Clinical significance of colonic fermentation." American Journal of Gastroenterology **85**(10): 1307-1312.
Ruff, W. E. and M. A. Kriegel (2015). "Autoimmune host-microbiota interactions at barrier sites and beyond." Trends Mol Med **21**(4): 233-244.
Ruiz, L., P. Ruas-Madiedo, et al. (2011). "How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences." Genes and Nutrition **6**(3): 307-318.
Russell, S. L., M. J. Gold, et al. (2012). "Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma." EMBO Reports **13**(5): 440-447.
Saavedra, J., A. Tschernia, et al. (1999). "Gastrointestinal function in infants consuming a weaning food supplemented with oligofructose, a prebiotic." Journal of pediatric gastroenterology and nutrition **29**(4): 513.
Saha, D. C. and R. A. Reimer (2014). "Long-term intake of a high prebiotic fiber diet but not high protein reduces metabolic risk after a high fat challenge and uniquely alters gut microbiota and hepatic gene expression." Nutrition Research **34**(9): 789-796.

Sajilata, G., R. S. Singhal, et al. (2002). "Weaning Foods: A Review of the Indian Experience." Food and Nutrition Bulletin **23**(2): 208-226.
Sajilata, M. G., R. S. Singhal, et al. (2006). "Resistant Starch–A Review." Comprehensive Reviews in Food Science and Food Safety **5**(1): 1-17.
Sandberg, A.-S. and T. Andlid (2002). "Phytogenic and microbial phytases in human nutrition." International Journal of Food Science & Technology **37**(7): 823-833.
Sandstrom, B., A. Cederblad, et al. (1986). "Retention of zinc and calcium from the human colon." Am J Clin Nutr **44**(4): 501-504.
Sandstrom, B., L. Davidsson, et al. (1990). "Selenium status and absorption of zinc (65Zn), selenium (75Se) and manganese (54Mn) in patients with short bowel syndrome." Eur J Clin Nutr **44**(10): 697-703.
Scheiwiller, J., E. Arrigoni, et al. (2006). "human fecal microbiota develops the ability to degrade type 3 resistant starch during weaning." journal of pediatric gastroenterology and nutrition **43**: 584-591.
Scheppach, W. (1994). "Efects of short chain fatty acids on gut morphology and function." Gut **S1**(1 Suppl): S35-38.
Scholtens, P. A., M. S. Alles, et al. (2006). "Bifidogenic effects of solid weaning foods with added prebiotic oligosaccharides: a randomised controlled clinical trial." J Pediatr Gastroenterol Nutr **42**(5): 553-559.
Scholtens, P. A., R. Oozeer, et al. (2012). "The early settlers: intestinal microbiology in early life." Annu Rev Food Sci Technol **3**: 425-447.
Scholz-Ahrens, K. E., G. Schaafsma, et al. (2001). "Effects of prebiotics on mineral metabolism." Am J Clin Nutr **73**(2 Suppl): 459S-464S.
Scott, K. P., S. W. Gratz, et al. (2013). "The influence of diet on the gut microbiota." Pharmacol Res **69**(1): 52-60.
Seal, C. and J. C. Mathers (1989). "Intestinal zinc transfer by everted gut sacs from rats given diets containing different amounts and types of dietary fibre." British Journal of Nutrition **62**: 151-163.
Sellitto, M., G. Bai, et al. (2012). "Proof of Concept of Microbiome-Metabolome Analysis and Delayed Gluten Exposure on Celiac Disease Autoimmunity in Genetically At-Risk Infants." PLoS One **7**(3): e33387.
Sepehri, S., R. Kotlowski, et al. (2007). "Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease." Inflamm Bowel Dis **13**(6): 675-683.
Shanks, N., R. Greek, et al. (2009). "Are animal models predictive for humans?" Philosophy, Ethics, and Humanities in Medicine : PEHM **4**: 2-2.
Shepherd, S. J., M. C. E. Lomer, et al. (2013). "Short-Chain Carbohydrates and Functional Gastrointestinal Disorders." American Journal of Gastroenterology **108**(5): 707-717.
Shulman, R. J., W. W. Wong, et al. (1983). "Utilization of dietary cereal by young infants." J Pediatr **103**(1): 23-28.
Singh, J., A. Dartois, et al. (2010). "Starch digestibility in food matrix: a review." Trends in Food Science & Technology **21**(4): 168-180.
Singh, V., J. Yang, et al. (2014). "Translating molecular physiology of intestinal transport into pharmacologic treatment of diarrhea: stimulation of na(+) absorption." Clin Gastroenterol Hepatol **12**(1): 27-31.
Singh, V., J. Yang, et al. (2014). "Translating molecular physiology of intestinal transport into pharmacologic treatment of diarrhea: stimulation of Na+ absorption." Clin Gastroenterol Hepatol **12**(1): 27-31.
Slavin, J. (2013). "Fiber and Prebiotics: Mechanisms and Health Benefits." Nutrients **5**(4): 1417-1435.
Smilowitz, J. T., C. B. Lebrilla, et al. (2014). "Breast milk oligosaccharides: structure-function relationships in the neonate." Annu Rev Nutr **34**: 143-169.
Smith, J. C., Jr., E. G. McDaniel, et al. (1972). "Effect of microorganisms upon zinc metabolism using germfree and conventional rats." J Nutr **102**(6): 711-719.
Smith, P., M. Howitt, et al. (2013). "The microbial metabolites, short chain fatty acids, regulate colonic Treg cell homeostasis." Science (New York, N.Y.) **341**(6145):

10.1126/science.1241165.
Sonnenburg, E. D. and J. L. Sonnenburg (2014). "Starving our Microbial Self: The deleterious Consequences of a diet deficient in microbiota-accessible carbohydrates." Cell Metabolism **20**: 779-786.
Sorensen, J. A., O. Andersen, et al. (1998). "An in vivo study of the gastrointestinal absorption site for zinc chloride in mice." J Trace Elem Med Biol **12**(1): 16-22.
Stanbury, P. F., A. Whitaker, et al. (1995). CHAPTER 1 - An Introduction to Fermentation Processes. Principles of Fermentation Technology (Second Edition). Amsterdam, Pergamon**:** 1-11.
Stark, P. L. and A. Lee (1982). "The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life." J Med Microbiol **15**(2): 189-203.
Starke, I. C., R. Pieper, et al. (2014). "The impact of high dietary zinc oxide on the development of the intestinal microbiota in weaned piglets." FEMS Microbiology Ecology **87**(2): 416-427.
Subramanian, S., L. V. Blanton, et al. (2015). "Cultivating healthy growth and nutrition through the gut microbiota." Cell **161**(1): 36-48.
Subramanya, S. B., V. M. Rajendran, et al. (2007). "Differential regulation of cholera toxin-inhibited Na-H exchange isoforms by butyrate in rat ileum." Am J Physiol Gastrointest Liver Physiol **293**(4): G857-863.
Symon, B. and M. Bammann (2012). "Feeding in the first year of life Emerging benefits of introducing complementary solids from 4 months." Australian Family Physician **41**: 226-229.
Symonds, E. L., S. Kritas, et al. (2004). "A combined 13CO2/H2 breath test can be used to assess starch digestion and fermentation in humans." J Nutr **134**(5): 1193-1196.
Szajewska, H., A. Guarino, et al. (2014). "Use of probiotics for management of acute gastroenteritis: a position paper by the ESPGHAN Working Group for Probiotics and Prebiotics." J Pediatr Gastroenterol Nutr **58**(4): 531-539.
Tachon, S., J. Zhou, et al. (2013). "The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses." FEMS Microbiology Ecology **83**(2): 299-309.
Taneja, D. K. and A. Malik (2014). "Zinc: An Effective but Neglected Child Survival Intervention." Indian Journal of Community Medicine : Official Publication of Indian Association of Preventive & Social Medicine **39**(4): 191-193.
Tang, M. K. and C. J. Lodge (2016). "EXamining the evidence for using synbiotics to treat or prevent atopic dermatitis." JAMA Pediatrics **170**(3): 201-203.
Taylor, C. M., J. R. Bacon, et al. (1991). "Homeostatic regulation of zinc absorption and endogenous losses in zinc-deprived men." Am J Clin Nutr **53**(3): 755-763.
Tetlow, I. J. and M. J. Emes (2011). 4.04 - Starch Biosynthesis in Higher Plants: The Starch Granule A2 - Moo-Young, Murray. Comprehensive Biotechnology (Second Edition). Burlington, Academic Press**:** 37-45.
Theil, E. C. (2011). "Iron homeostasis and nutritional iron deficiency." J Nutr **141**(4): 724S-728S.
Thum, C., A. L. Cookson, et al. (2012). "Can nutritional modulation of maternal intestinal microbiota influence the development of the infant gastrointestinal tract?" J Nutr **142**(11): 1921-1928.
Topping, D. L. (2016). "Targeted delivery of short-chain fatty acids to the human large bowel." The American Journal of Clinical Nutrition **104**(1): 1-2.
Topping, D. L. and P. M. Clifton (2001). "Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides." Physiol Rev **81**(3): 1031-1064.
Turnbaugh, P. J., R. E. Ley, et al. (2006). "An obesity-associated gut microbiome with increased capacity for energy harvest." Nature **444**(7122): 1027-1031.
Umar, S. (2010). "Intestinal Stem Cells." Curr Gastroenterol Rep **12**(5): 340-348.
Umu, O. C., J. A. Frank, et al. (2015). "Resistant starch diet induces change in the swine microbiome and a predominance of beneficial bacterial populations." Microbiome **3**: 16.
Van den Abbeele, P., P. Gerard, et al. (2011). "Arabinoxylans and inulin differentially modulate the mucosal and luminal gut microbiota and mucin-degradation in humanized rats." Environ Microbiol **13**(10): 2667-2680.
Van der Maarel, M. J. and H. Leemhuis (2013). "Starch modification with microbial alpha-glucanotransferase enzymes." Carbohydr Polym **93**(1): 116-121.

van Stuijvenberg, M., A. M. Eisses, et al. (2011). "Do prebiotics reduce the number of fever episodes in healthy children in their first year of life: a randomised controlled trial." Br J Nutr **106**(11): 1740-1748.
Vandenplas, Y., I. Zakharova, et al. (2015). "Oligosaccharides in infant formula: more evidence to validate the role of prebiotics." Br J Nutr **113**(9): 1339-1344.
Venema, K. and P. van den Abbeele (2013). "Experimental models of the gut microbiome." Best Pract Res Clin Gastroenterol **27**(1): 115-126.
Venkataraman, A., J. R. Sieber, et al. (2016). "Variable responses of human microbiomes to dietary supplementation with resistant starch." Microbiome **4**(1): 33.
Ventura, M., F. Turroni, et al. (2015). Chapter 4 - Bifidobacteria of the Human Gut: Our Special Friends. Diet-Microbe Interactions in the Gut. K. T. D. Rio. San Diego, Academic Press**:** 41-51.
Verity, K. and C. Edwards (1994). "Resistant starch in young children." Proc Nutr Soc **53**: 105.
Vigsnaes, L. K., J. Holck, et al. (2011). "In vitro fermentation of sugar beet arabino-oligosaccharides by fecal microbiota obtained from patients with ulcerative colitis to selectively stimulate the growth of Bifidobacterium spp. and Lactobacillus spp." Appl Environ Microbiol **77**(23): 8336-8344.
Viveros, A., A. Brenes, et al. (2002). "Effects of Microbial Phytase Supplementation on Mineral Utilization and Serum Enzyme Activities in Broiler Chicks Fed Different Levels of Phosphorus." Poultry Science **81**(8): 1172-1183.
Vohra, A. and T. Satyanarayana (2003). "Phytases: microbial sources, production, purification, and potential biotechnological applications." Crit Rev Biotechnol **23**(1): 29-60.
Vonk, R. J., R. E. Hagedoorn, et al. (2000). "Digestion of so-called resistant starch sources in the human small intestine." Am J Clin Nutr **72**(2): 432-438.
Voreades, N., K. Anne, et al. (2014). "Diet and the development of the human intestinal microbiome." Front Microbiol **5**.
Walker, A. W., S. H. Duncan, et al. (2005). "pH and Peptide Supply Can Radically Alter Bacterial Populations and Short-Chain Fatty Acid Ratios within Microbial Communities from the Human Colon." Applied and environmental microbiology **71**(7): 3692-3700.
Walker, A. W., J. Ince, et al. (2011). "Dominant and diet-responsive groups of bacteria within the human colonic microbiota." ISME J **5**(2): 220-230.
Walker, C. L. and R. E. Black (2010). "Zinc for the treatment of diarrhoea: effect on diarrhoea morbidity, mortality and incidence of future episodes." Int J Epidemiol **39 Suppl 1**: i63-69.
Walker, C. L. F., I. Rudan, et al. (2013). "Global burden of childhood pneumonia and diarrhoea." The Lancet **381**(9875): 1405-1416.
Walter, J., C. Hertel, et al. (2001). "Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis." Appl Environ Microbiol **67**(6): 2578-2585.
Wang, L., C. T. Christophersen, et al. (2012). "Elevated fecal short chain fatty acid and ammonia concentrations in children with autism spectrum disorder." Dig Dis Sci **57**(8): 2096-2102.
Wang, Q., G. M. Garrity, et al. (2007). "Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy." Appl Environ Microbiol **73**(16): 5261-5267.
Wang, X., P. L. Conway, et al. (1999b). "In vitro utilization of amylopectin and high-amylose maize (amylomaize) starch Granules by Human Colonic Bacteria." Applied and environmental microbiology **65**: 4848-4854.
Ward, D. M., R. Weller, et al. (1990). "16S rRNA sequences reveal numerous uncultured microorganisms in a natural community." Nature **345**(6270): 63-65.
Wei-Liang Tan, J., C. Valerio, et al. "A randomized trial of egg introduction from 4 months of age in infants at risk for egg allergy." Journal of Allergy and Clinical Immunology **139**(5): 1621-1628.e1628.
Wessels, K. and K. H. Brown (2012). "Estimating the global prevalence of Zinc Deficiency." PLOS ONe **7**(11): 1-11.
West, C. E., H. Renz, et al. (2015). "The gut microbiota and inflammatory noncommunicable diseases: Associations and potentials for gut microbiota therapies." J Allergy Clin Immunol **135**(1): 3-13.

Whitton, C., S. K. Nicholson, et al. (2011). "National Diet and Nutrition Survey: UK food consumption and nutrient intakes from the first year of the rolling programme and comparisons with previous surveys." Br J Nutr **106**(12): 1899-1914.
WHO (2005). The Treatment of Diarrhoea: a Manual for Physicians and Other Senior Health Workers. Geneva, Switzerland, World Health Organization (WHO).
Willemsen, L. E. M., M. A. Koetsier, et al. (2003). "Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts." Gut **52**(10): 1442-1447.
Williams, C. F., G. E. Walton, et al. (2015). "Comparative analysis of intestinal tract models." Annu Rev Food Sci Technol **6**: 329-350.
Wise, A. and D. Gilburt (1982). "Phytate hydrolysis by germfree and conventional rats." Applied and environmental microbiology **43**(4): 753-756.
Wolf, B. W., L. L. Bauer, et al. (1999). "Effects of Chemical Modification on in Vitro Rate and Extent of Food Starch Digestion: An Attempt To Discover a Slowly Digested Starch." Journal of Agricultural and Food Chemistry **47**(10): 4178-4183.
Woolnough, J. W., J. A. Monro, et al. (2008). "Simulating human carbohydrate digestionin vitro: a review of methods and the need for standardisation." International Journal of Food Science & Technology **43**(12): 2245-2256.
Wu, H., V. Tremaroli, et al. (2015). "Linking Microbiota to Human Diseases: A Systems Biology Perspective." Trends Endocrinol Metab **26**(12): 758-770.
Yang, J., Y. Zhang, et al. (2013). "Gene profile identifies zinc transporters differentially expressed in normal human organs and human pancreatic cancer." Curr Mol Med **13**(3): 401-409.
Yao, Y., D. B. Thompson, et al. (2004). "Maize Starch-Branching Enzyme Isoforms and Amylopectin Structure. In the Absence of Starch-Branching Enzyme IIb, the Further Absence of Starch-Branching Enzyme Ia Leads to Increased Branching." Plant Physiology **136**(3): 3515-3523.
Yatsunenko, T., F. E. Rey, et al. (2012). "Human gut microbiome viewed across age and geography." Nature **486**(7402): 222-227.
Yonekura, L. and H. Suzuki (2005). "Effects of dietary zinc levels, phytic acid and resistant starch on zinc bioavailability in rats." Eur J Nutr **44**(6): 384-391.
Younes, H., C. Demigne, et al. (1996). "Acidic fermentation in the caecum increases absorption of calcium and magnesium in the large intestine of the rat." British Journal of Nutrition **75**: 301-314.
Young, W., N. C. Roy, et al. (2012). "Changes in bowel microbiota induced by feeding weanlings resistant starch stimulate transcriptomic and physiological responses." Appl Environ Microbiol **78**(18): 6656-6664.
Yue, P. and S. Warning (1998). "Functionality of resistant starch in food applications." Food Australia **50**: 615-621.
Zacharias, B., H. J. Lantzsch, et al. (1999). "Influence of microbial phytase and dietary calcium on the accumulation of lead in different organs of pigs." Biological Trace Element Research **70**(3): 243.
Zaman, S. A. and S. R. Sarbini (2015). "The potential of resistant starch as a prebiotic." Crit Rev Biotechnol: 1-7.
Ze, X., S. H. Duncan, et al. (2012). "Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon." ISME J **6**(8): 1535-1543.
Zeeman, S. C., J. Kossmann, et al. (2010). "Starch: its metabolism, evolution, and biotechnological modification in plants." Annu Rev Plant Biol **61**: 209-234.