University of Reading

The effect of prebiotics, probiotics, synbiotics and zinc carnosine on bacterial metabolism in an *in vitro* gut model fermentation system

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Simone Pyle, 2021

S.Pyle

Acknowledgement

‘Success is not final, failure is not fatal: it is the courage to continue that counts.’ This quote by Winston Churchill sums up my PhD journey. It has not been easy as there have been times where I wanted to give up and others where I loved every second of it. My knowledge of the area has advanced but I have also learnt so much about myself.

Getting to this stage is one of my proudest achievements especially since I am dyslexic and writing is not my strongest quality. The people who have supported me along the way have provided me with the determination, perseverance and ambition to do well throughout my PhD and pursue my career objectives.

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1 Table of Contents

2 Abstract

In Western populations up to one in four individuals meet the criteria for functional gastrointestinal disorder. Modulation of the gut microbiota composition and bacterial metabolism may improve this and the main driver for these alterations is through dietary components. Prebiotics, probiotics and synbiotics have previously been shown to modulate the gut microbiota. Therefore the aim was to investigate the bacterial metabolism following a wheat dextrin, partially hydrolysed guar gum, inulin, *Bifidobacterium lactis* HN019, *Lactobacillus acidophilus* NCFM and zinc carnosine intervention in an *in vitro* batch culture and three stage continuous culture gut model system. A secondary aim was to investigate impact of the interventions on Irritable Bowel Syndrome-Diarrhoea and compare this to healthy donors. The third aim was to assess the impact of zinc carnosine on the gut microbiota. Samples were collected at multiple time points and used to enumerate bacteria through fluorescence *in situ* hybridisation flow-cytometry, organic acid production via gas chromatography and neurotransmitter gamma-aminobutyric acid through liquid chromatography-mass spectrometry. The main findings were that partially hydrolysed guar gum and wheat dextrin were metabolised by *Bacteroides* and *Clostridium* cluster IX and produced propionate with partially hydrolysed guar gum being fermented to a greater extent. The addition of a probiotic strain led to no changes in bacterial metabolism. Inulin led to an increase in *Bifidobacterium* spp., which was prolonged by a synbiotic. The production of gamma-aminobutyric acid was significantly increased in healthy donor inocula following prebiotic and synbiotic interventions but this was not seen in the Irritable Bowel Syndrome-Diarrhoea group. Additionally, zinc carnosine may promote *Bifidobacterium* spp., and lactic acid bacteria but further research is required as some bacterium in the *Clostridium histolyticum* group are pathogenic and these were also elevated. Overall, the prebiotic had a greater impact on the gut microbiota although,

synbiotics did have positive influences on the gut microbiota of both healthy and Irritable Bowel Syndrome-Diarrhoea donors.

3 Abbreviations

°C	Degrees celsius
2'FL	2'-fucosyllactose
ALA	α-linolenic acid
ANOVA	Analysis of variance
BMI	Body mass index
$CaCl_2$	Calcium chloride
CFU/mL	Colony forming units per millilitre
CLA	Conjugated linoleic acid
CNS	Central nervous system
CO_2	Carbon dioxide
CRP	C-reactive protein
DP	Degree of polymerisation
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EC	Enterochromaffin
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ENS	Enteric nervous system
EPA	Eicosapentaenoic acid
FISH-FCM	Fluorescence *in situ* hybridisation flow-cytometry
FODMAPS	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
FOS	Fructooligosaccharide
G	Gram
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
GM	Gut model
GOS-3	Galactose-galactose-glucose trisaccharide
GOS-4	Galactose-galactose-galactose-glucose tetrasaccharide
GPCRs	G-coupled receptors
h	Hours
HDL	High-density lipoproteins
HN019	*Bifidobacterium lactis* HN019
HMOs	Human milk oligosaccharides
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IBS-A	Irritable Bowel Syndrome Alternating
IBS-C	Irritable Bowel Syndrome Constipation
IBS-D	Irritable Bowel Syndrome Diarrhoea
IFN-γ	Interferon gamma
IL	Interleukin

K_2HPO_4	Dipotassium hydrogen phosphate
KH_2PO_4	Monopotassium phosphate
L	Litre
LCMS	Liquid chromatography–mass spectrometry
LDL	Low-density lipoproteins
LPS	Lipopolysaccharides
LNnT	lacto-N-neotetraose
Mg	Miligram
$MgSO_4$	Magnesium sulfate
ML	Mililitres
mM	Milimolar
MRS	Man-Rogosa-Sharpe
MRS-C	Man-Rogosa-Sharpe with L-cysteine
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NCFM	*Lactobacillus acidophilus* NCFM
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	natural killer cells
O_2	Oxygen
OD	Optical density
PBS	Phosphate buffer solution
PFA	Paraformaldehyde
pH	Potential of hydrogen
PHGG	Partially hydrolysed guar gum
PUFA	Polyunsaturated fatty acid
PUSH	Pressure Ulcer Scale for Healing
PYY	peptide YY
SCFA	Short-chain fatty acid
SD	Standard deviation
SDA	stearidonic acid
SPSS	Statistical package for the social
SS	Steady state
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells
UC	Ulcerative Colitis
V	Vessel
WD	Wheat dextrin
Xg	Relative centrifugal force
XOS	Xylooligosaccharide
ZnC	Zinc carnosine
Zur	Zinc uptake regulator
µl	Microlitres

1 Chapter 1: Human gut microbiota and the influence of probiotics, prebiotics and micronutrients

In press as a book chapter in Comprehensive Gut Microbiota, Elsevier book

1.1 Abstract

Dietary supplementation can modulate the gut microbiota leading to positive effects on host health. This chapter outlines the most commonly used approaches: prebiotics, probiotics, synbiotics and micronutrients. Each section describes the different types of interventions, how they modulate the gut microbiota and scientific evidence on how supplementation affects certain disorders and/or diseases.

Key words: *Bifidobacterium*, FOS, GOS, gut microbiota, inulin, *Lactobacillus*, minerals, prebiotic, probiotic, synbiotic and vitamins

1.2 Introduction

Chronic diseases including cardiovascular complaints, Type II diabetes, many cancers, some dementias, acute and chronic gut disorders are major and growing societal and financial concerns. Moreover, an increasingly obese and ageing population means there is greater prevalence of a range of disorders. Today's health model comprises of both preventative life style and therapeutic entities, with diet playing a principal role. Many functional foods in use are targeted at gastrointestinal health, with the indigenous microbiota being seen as critical in health sustenance. The human gastrointestinal microbiome varies markedly in composition and activity depending upon anatomical area under consideration. By far, the majority of gut microorganisms reside in the large intestine. Many factors can affect the gut microbiota such as genetics, age, diet, physiological state and microbial interaction (Turnbaugh and Gordon, 2009). One factor which has a rapid effect and major impact on the gut microbiota is diet. One

study demonstrating the ability of diet alter the gut microbiome is David et al. (2014) which shows alteration to an individual's habitual diet can lead to a shift in bacterial communities within 24 hours.

1.3 Prebiotics

The western diet consists of a low amount of fibre compared to many other diets and it has been reported that the majority of the United Kingdom population do not consume the recommended 30g of fibre per day (Bindels et al., 2015, Hooper et al., 2015). Fibre can be found in many plant-based foods such as vegetables, fruit, legumes, grains and seeds (Cai et al., 2020). Dietary fibre comprises of non-digestible carbohydrates which are not fully absorbed in the small intestine therefore, reach the large intestine, where indigenous microbes can metabolise them. Prebiotics are a derivative of fibres.

Driven by the increasing burden of gastrointestinal disease, the functional foods market has moved heavily towards gut derived events. Specifically, these target the human gut to stimulate beneficial microbial genera. Prebiotics serve to elicit changes in the gut microbiota composition that increase populations of purported beneficial bacterial genera, for example, bifidobacteria. They are non-viable food components (carbohydrates) that have a selective microbial metabolism in the human gut. They attempt to induce beneficial changes by specifically fortifying levels of certain bacteria indigenous to the gut microbiota. As such, selectivity of their fermentation is key.

The term prebiotic has evolved over the years with the first definition being 'A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (Gibson and Roberfroid, 1995). The most recently updated definition is 'A substrate that is selectively utilised by host microorganisms conferring a health benefit'

(Gibson et al., 2017). The new definition takes into account the connection between multiple organs, including the gut-brain axis, and it allows a greater scope for beneficial affects across the body not restricted to just the gut i.e. including skin, the oral cavity, urogenital areas. The latest definition also introduces new substrates to be included as prebiotics, for example conjugated linoleic acid (CLA), polyunsaturated fatty acid (PUFA), Human milk oligosaccharides (HMOs), Phenolics and phytochemicals (Gibson et al., 2017).

1.3.1 Prebiotic oligosaccharides

1.3.1.1 Inulin and Fructooligosaccharides

A well-studied and positive prebiotic recognised to selectively stimulate bifidobacteria (seen as a health positive genus) is inulin, a type of fructooligosaccharide (Gibson and Roberfroid, 1995). Inulin consists of d-fructose joined by ß-(2 → 1) linkage and can be found in chicory and Jerusalem artichoke (Roberfroid, 2005). The degree of polymerisation (DP) of inulin averages at 12 (but can be found at DP between 2 to 60) (Roberfroid, 2005).

Short chain fructooligosaccharide (FOS), also known as oligofructose, has a lower DP <10 (average DP between 2 to 8) as it is partially hydrolysed inulin which results in it being more readily fermentable. FOS contains β-(2,1) fructose chains with terminal glucose units (Flickinger et al., 2003).

FOS are used within the food industry by incorporating them in products to reduce sugar and fat contents. Long chain inulin is slightly soluble, viscous and forms microcrystals when sheared in water and milk, resulting in a creamy texture therefore being ideal as a fat replacer for dairy products (Franck, 2007, Lopez-Molina et al., 2005). Inulin is also used as a bulking agent and thickener in sauces, spreads, chocolate and ice-cream (Yi et al., 2009). Low molecular weight fructans are soluble and sweet being ideal as a sugar replacement in foods, especially due to them being calorie free as it is not absorbed in the upper gut but degraded by

bacteria in the large intestine (Franck, 2007). Inulin and FOS can be used as supplements that are added to water, tea and coffee.

In a small study of 4 participants it was reported that feeding 15g of inulin or FOS per day for 15 days led to a significant increase in *Bifidobacterium* (FOS increased by 0.7 $\log_{10}$ g stool $^{-1}$ and inulin increased by 0.9 $\log_{10}$ g stool $^{-1}$) (Gibson et al., 1995). A larger study of 30 healthy participants showed a significant increase in bifidobacteria levels after 2 weeks of either a low dose of inulin (5g) and high dose (8g) (Kolida et al., 2007). A double-blind randomised control trial outlined that consumption of Jerusalem artichoke inulin or chicory inulin (7.7g per day) resulted in an increase in bifidobacteria and reduction in *Bacteroides-Prevotella* and *Clostridium histolyticum* (Kleessen et al., 2007). Likewise, the same was described in 32 healthy adults after consuming inulin at a dose of 10g per day for 3 weeks (Costabile et al., 2010). Ramirez-Farias et al. (2009) concluded that the most prevalent species of *Bifidobacterium* after an inulin intervention (12 participants consumed 10g of inulin per day) were *B. adolescentis* and *B. bifidum.*

Similar to inulin, 8 participants with supplementation of 8g of FOS per day also showed selectivity for bifidobacteria after 2 weeks and this continued until the end of the intervention period (5 weeks) (Menne et al., 2000). A larger study using FOS as an intervention resulted in an increase in bifidobacteria after a 2 month intervention in 61 healthy formula fed infants (Paineau et al., 2014). Another study in 56 infants showed that low doses (FOS, 0.4g/d per day) for 14 days added to formula feed led to a significant increase in bifidobacteria compared to the placebo group (maltodextrin) (Kapiki et al., 2007). An adult study assessing impacts of varies doses (2.5g, 5g, 7.5g and 10g per day) of short chain FOS showed that all doses increased in count of bifidobacteria when plated compared to the placebo (Bouhnik et al., 2006).

A typical dose of inulin required to selectively stimulate for *Bifidobacterium* is 5 g per day and the majority of studies use doses between 5-15g per day, which are well-tolerated

(Kolida et al., 2007, Bonnema et al., 2010). Whereas, supplementation of FOS at 10g per day resulted in adverse events showing some gastrointestinal symptoms which may be due to the shorter chain lengths being more readily fermentable therefore a lower dose may be more effective as a FOS intervention (Bonnema et al., 2010).

1.3.1.2 Galactooligosaccharides

Galactooligosaccharides are produced from lactose by β-galactosidase in a kinetic controlled reaction between enzymatic hydrolysis and transgalactosylation (Torres et al., 2010). Firstly, a lactose molecule binds to β -galactosidase and forms galactosyl-enzyme complex releasing a glucose molecule. Secondly, the intermediated react with another lactose molecule to form gal-gal-glu trisaccharide (GOS-3) and the process continues forming GOS-4 (Vera et al., 2016).

GOS was originally used as a substitute for human milk-oligosaccharides (HMOs) for newborns in Japan in 1970 (Vera et al., 2016). An increase in bifidobacteria was shown in healthy infants after consuming formula feed supplemented with GOS (Sierra et al., 2015, Ben et al., 2008, Matsuki et al., 2016). A combination of FOS and GOS added to formula feed has been reported to be similar to breast milk with an increase in SCFA resulting in comparable pH levels (Knol et al., 2005, Veereman-Wauters et al., 2011). More recently, GOS has been used in food products due to its ability to withstand high temperatures and low pH (Sako et al., 1999). Studies have shown fermentation of GOS to significantly increase *Bifidobacterium* spp. in the gut e.g. Davis et al. (2011) showed up to a tenfold increase in bifidobacteria after consuming GOS (10g/day) for 3 weeks with a dose response-relationship being found. Similar to inulin, a minimum dose of 5g per day is required to stimulate the growth of bifidobacteria (Davis et al., 2010). In elderly participants, GOS at a dose of 5.5g per day for 10 weeks increased bifidobacteria and bacteroides (Vulevic et al., 2015a).

1.3.1.3 Xylooligosaccharide

Xylooligosaccharide (XOS) is a much less researched prebiotic compared to FOS or GOS (Gibson et al., 2017). It is made up of xylose units and are sugar oligomers (Vázquez et al., 2000). They are usually found naturally in fruits, vegetables, milk, bamboo shoots and honey (Vázquez et al., 2000). An *in vitro* batch culture study found XOS had the ability to increase the growth of *Bifidobacterium* (Carlson et al., 2017). The dose required is less than the other oligosaccharides mentioned above as 1g per day has been show to stimulate bifidobacteria in healthy adults although, a dose response study showed a higher increase in bifidobacteria with higher doses of XOS (up to 2.8g) (Okazaki et al., 1990, Finegold et al., 2014).

1.3.2 Health benefits of prebiotic oligosaccharides

1.3.2.1 Gut health and gastrointestinal diseases/disorders

The classification for a prebiotic requires "conferring a health benefit" (Gibson et al., 2017). Numerous randomised controlled trials have been carried out assessing prebiotic supplement and have found positive health outcomes (Azpiroz et al., 2017, Dehghan et al., 2014a, Drakoularakou et al., 2010). Inulin is known to help regulate bowel movements relieving constipation and has been accepted as a health claim by European Food Safety Authority (EFSA), "Chicory inulin contributes to maintenance of normal defecation by increasing stool frequency" (EFSA, 2015, Hond et al., 2000).

Prebiotics have been seen to alleviate gastrointestinal symptoms such as constipation and reduce diarrhoea. Improvement in stool frequency, consistency and softer stool was observed following consumption of inulin in children, adults and elderly individuals with constipation, through a double-blind randomised, control-placebo trial (Closa-Monasterolo et al., 2017, Micka et al., 2017, Marteau et al., 2011).

Prebiotics can also help relieve Travellers' diarrhoea which occurs when travelling from a location of high hygiene levels to a lower one and often occurs when individuals consume contaminated water or food (Black, 1990). In a randomised control trial, 159 healthy volunteers consumed either GOS or placebo (maltodextrin) a week prior to traveling aboard and throughout the duration of their holiday (Drakoularakou et al., 2010). There is a lower incidence of travellers' diarrhoea in participants who consumed GOS and the duration of diarrhoea was almost halved (Drakoularakou et al., 2010). End-products of prebiotic fermentation are short chain fatty acids (SCFA) these can also benefit individuals with travellers' diarrhoea. In sixty volunteers who consumed sodium butyrate and other organic acids three days prior to travel, and during holiday, a significant reduction in diarrhoea symptoms, number of stools per day and GI symptoms occurred (pain, bloating, nausea and vomiting) (Krokowicz et al., 2014).

Oligosaccharides can also have a major role to play in improving chronic gastrointestinal diseases and disorders. Inflammatory bowel disease (IBD) is chronic inflammation of the gastrointestinal tract and is a relapsing disorder (Ghouri et al., 2014). The pathophysiology is unknown but involves the immune response and is often treated with anti-inflammatory prescriptions (Ghouri et al., 2014). It encompasses ulcerative colitis and Crohn's disease. In volunteers with ulcerative colitis, consumption of 12g per day of inulin and FOS combined led to a reduction in calprotectin, a protein biomarker used to indicate intestine inflammation, and dyspeptic symptoms (Casellas et al., 2007).

Irritable bowel syndrome (IBS) is a common functional GI disorder that has been defined as 'chronic, relapsing and often lifelong and is characterised by abdominal pain or discomfort which may be associated with defaecation and/or accompanied by a change in bowel habits' (NICE, 2008). Symptoms of IBS include stomach cramps, bloating, diarrhoea, constipation, increased gut sensitivity and problems with digestion (Dupont, 2014, Ringel et

al., 2011). The disorder is separated into three types: IBS-Diarrhoea (IBS-D), IBS-Constipation (IBS-C) and IBS-Alternating (IBS-A). There is evidence to suggest prebiotics can help to reduced symptoms of IBS, as in 42 volunteers, GOS supplementation was effective in reducing flatulence, subjective abdominal pain, bloating and bowel movement difficulties and improved stool consistency (Silk et al., 2009). Additionally, in IBS-D volunteers a significant anxiety score was found and this also decreased using 7g per day of B-GOS supplementation (Silk et al., 2009). A randomised double blind study found that after 4 weeks consuming short-chain FOS (5g per day) a reduced anxiety score was shown compared to the placebo group and in IBS-C volunteers a decrease in renal sensitivity occurred (Azpiroz et al., 2017). In another randomised control trial IBS symptoms worsened after consumption of FOS, however, a high dose of 20g per day was consumed (Olesen and Gudmand-Høyer, 2000).

1.3.2.2 Immune function

The immune system is complex and the gut microbiota interacts in a range of ways with the immune system via bacterial communities, fermentation end-products, host receptors, signalling molecules and molecular pathways (Lazar et al., 2018). Fermentation of inulin and FOS can lead to alterations in the immune response but there is limited research in this area (Fung et al., 2012).

Inulin and FOS specifically increase the growth of bifidobacteria and to a lesser extent lactobacilli, these two genera have antimicrobial properties which have been shown to reduce/inhibit the growth of pathogenic bacteria such as *Clostridium perfringens, E. coli*, *Campylobacter jejuni* and *Salmonella enteritidis* (Gibson and Roberfroid, 1995, Kleessen et al., 1997, Fooks and Gibson, 2002). A reduction in pathogenic bacteria after the fermentation of FOS may also induce a reduction in inflammation by reducing exotoxins (lipopolysaccharides) (Watzl et al., 2005). It has also been found that pro-inflammatory

cytokines were reduced following a prebiotic intervention, where through a human intervention trial of 8g of FOS per day for 3 weeks decreased interleukin 6 (IL-6) (Guigoz et al., 2002).

In addition, inulin and FOS fermentation results in SCFA production which may affect the immune response. SCFA lower luminal pH which may be a mechanism used to inhibit the growth of pathogenic bacteria (Macfarlane and Gibson, 1997). The SCFA butyrate provides energy to the colon, helping to regulate cell growth and differentiation, improving integrity of the mucosal barrier and regulating immunity, therefore altering physiological properties of the colon (Cook and Sellin, 1998, Fung et al., 2012).

More recent studies have claimed that, in patients with type 2 diabetes mellitus, which is associated with immune disruption and metabolic abnormalities, 10g/d of FOS and inulin supplementation for two months led to the reduction in pro-inflammatory markers IL-4, IL-12 and interferon gamma (IFN-γ) compared to a placebo group (Dehghan et al., 2016). Similarly, GOS supplementation in comparison to placebo control observed in forty elderly (65-80 years) volunteers documented an increase in anti-inflammatory IL-10, IL-8, natural killer cells (NK cells) and C-reactive protein (CRP) and a reduction in IL-1β (Vulevic et al., 2015b).

1.3.2.3 Metabolic syndrome

Metabolic syndrome is a collection of metabolic abnormalities which can increase the risk of developing strokes, coronary heart disease, cardiovascular risk and type 2 diabetes (Afsana et al., 2010). Some of the main risk factors are elevated high-density lipoproteins, total cholesterol, triglycerides, blood glucose, body mass index and systemic blood pressure and consumption of oligosaccharides have been found to improve these markers (Afsana et al., 2010). A randomised double blind cross-over study found that after consuming pasta enriched with 11% inulin, a reduction in triglycerides and cholesterol was shown (Russo et al., 2010). In overweight women with type 2 diabetes, it was found that after 10g of inulin a significant

reduction in fasting plasma glucose, lipopolysaccharides and inflammation markers IL-6 and tumor necrosis factor (TNF-α) compared to the control (maltodextrin) occurred (Dehghan et al., 2014a).

Lipopolysaccharides are found on the outside of Gram- negative bacteria cell walls and they can trigger an inflammatory response (Dehghan et al., 2014b). Gut microbiota may regulate metabolic endotoxemia therefore a dietary intervention may help to reduce obesity and type 2 diabetes where elevated levels of lipopolysaccharide occur (Cani et al., 2008). Prebiotics were shown to help weight management in a study by Cani et al. (2006) which found that feeding 16g per day of FOS resulted in increased satiety, reduced hunger and a 5% reduction in total energy intake in five healthy or overweight individuals. More recently, a larger (125 overweight or obese individuals) double-blind study combining inulin (2g per day) and FOS (6g per day) lead to reduced hunger and increased satiety (Reimer et al., 2017).

1.3.2.4 Mineral absorption

Most research into mineral absorption in the gut is on magnesium and calcium. Magnesium is key for bone metabolism, energy metabolism by glycolysis and other physiological functions (Lukaski and Nielsen, 2002). Calcium is important for blood coagulation, muscle contraction, nerve function and regulating bone mass (Gibson and Rastall, 2006, Roberfroid et al., 2010). Dietary fibre may play an important role in calcium absorption in the gut (Younes et al., 2007). In a randomised, double-blind, crossover design study in adolescents, it was shown that consuming 15g of oligofructose per day increased the fractional calcium absorption by 26% (Van den Heuvel et al., 1999). Another, adolescence study found a 12 % higher fractional calcium absorption compared to a control (15g total dietary fibre) after 3 weeks of consuming 27g per day of total dietary fibre (Whisner et al., 2014). Additionally, in fifteen postmenopausal

women, consumption of inulin and FOS combined for 6 weeks showed an increase in calcium and magnesium absorption and markers of bone turnover (Holloway et al., 2007). An increase in magnesium absorption was also found in postmenopausal women after 10g per day of FOS for 5 weeks (Tahiri et al., 2001).

Following fermentation of oligosaccharides, SCFA lowers the pH of the colon this can inhibit the ability of calcium to form complexes with metabolites such as phytates and oxalates (Whisner and Castillo, 2018). Additionally, this increases exchange of cellular hydrogen for luminal calcium and can also help with translocation (Lutz and Scharrer, 1991, Ohta et al., 1998, Raschka and Daniel, 2005). After fermentation of inulin and FOS combined there was a 2.2 fold larger surface area in the caecum of rats along with an increase in calcium availability (Raschka and Daniel, 2005).

1.3.3 Human milk oligosaccharides

Human milk has been associated with many health benefits such as reducing diarrhoea, fewer pneumonia incidence, decreased allergic reactions, improved gastrointestinal development, improved protection against chronic diseases in infants. It comprises bioactive compounds which influence immunity (Gdalevich et al., 2001, Owen et al., 2006). Human milk oligosaccharides (HMOs) naturally occur in human breast milk, where they are the third most abundant component after lactose and lipids, and are mainly made up of lactose, polylactosamine and lacto-N-biose core (Coppa et al., 1999, Kulinich and Liu, 2016). HMOs are considered prebiotics (Gibson et al., 2017). Infant formula feed typically does not contain HMOs therefore FOS and GOS have been used in these products to increase bifidobacteria. More recently, two structurally similar HMOs have been produced as commercial products: 2'-fucosyllactose (2'FL) and lacto-N-neotetraose (LNnT) (Bonner, 2019, Steenhout et al., 2016). These have been added to formula feeds producing a shift in the gut microbiota and

metabolites similar to that of human breast milk in comparison to formula feed without HMOs (Steenhout et al., 2016). Adding 2'FL and GOS to formula feed resulted in similar innate and adaptive changes to the immune system to a greater extent to adding GOS alone to infant feed (Goehring et al., 2016). However, this is early work and there are estimated to be a further 150 HMO compounds (Bonner, 2019).

1.3.4 Polyphenols

Polyphenols can be found in fruit, dry legumes, tea, coffee, red wine and fruit juice there are around 8000 different types which are categorised into flavonoids or non-flavonoids (Santhakumara et al., 2018, Bravo, 1998, Del Rio et al., 2013). Flavonoids are further split into Flavonols, Isoflavones, Anthocyanins, Flavan-3-ols, Proanthocyanidins and Flavones (Santhakumara et al., 2018). Non-flavonoids are broken into Phenolic acids, Hydrolysable tannins and Stilbenes (Santhakumara et al., 2018). They all have an aromatic ring with one or more hydroxyl group (Bravo, 1998, Del Rio et al., 2013). For phenolics and phytochemicals, 90-95% of them reach the colon where biotransformation occurs by the colonic bacteria (Bowey et al., 2003). There are many health benefits associated with polyphenols with cardiovascular disease being one of the most researched areas. A human trial reported after randomly assigning hypertensive participants to a low-polyphenol diet (< 2 portions of fruit and vegetables a week) or high polyphenol diet (one portion of berries per day and 50 g of dark chocolate) that participants in the high-polyphenol diet improved microvascular function and reduced cardiovascular disease risk (Noad et al., 2016). Research has also seen an improvement in insulin sensitivity following consumption of strawberry and cranberry polyphenols for 6 weeks in obese and overweight participants (non-diabetic) (Paquette et al., 2017). Grape and blueberry polyphenols have been found to improve memory of every day events (episodic

memory) in a subset of elderly participants (60-70 years old) who, at baseline, had declines in memory (Bensalem et al., 2019).

1.3.5 Conjugated linoleic acid

Many bacterial strains have been shown to convert linoleic acid to conjugated linoleic acid (CLA) which can have a positive impact on health. CLA cannot be synthesised by the body but can be produced by microbial fermentation. Usually, CLA is added as a supplement consisting of cis-9, trans-11 and trans-10, cis-12 isomers which is often called CLA 50:50 (Park et al., 1999). The majority of research has focused on the role CLA has to play in weight management through assessment of body mass index (BMI), body weight, lean body mass, body-fat mass and abdominal adiposity. A randomised double-blind, cross-over control trial found that when 55 obese type 2 diabetic women consumed CLA for 16 weeks a reduction in body mass index and total adipose occurred with no alteration in the control group (Safflower oil) (Norris et al., 2009). A study in fifty-three overweight, or obese, children found that three months of CLA supplement resulted in an increase in BMI but weight gain was not as high as in the placebo group (Racine et al., 2010). Additionally, this study found no difference in plasma glucose, insulin and low-density lipoprotein (LDL). This has been supported by other research showing no alteration in cardiovascular markers such as LDL, high-density lipoproteins (HDL), triacylglyceride and total cholesterol (Rubin et al., 2012, Tricon et al., 2006). However, there are some studies showing a positive effect. One recent study investigated the effect of pecorino cheese compared to CLA enriched pecorino cheese in 42 mild hypercholesterolaemia volunteers (Limongi et al., 2017). The study found that after 3 weeks consuming the enriched cheese (90g per day) a maintenance in LDL levels compared to baseline was found, as well as a smaller reduction in HDL compared to the control group (Limongi et al., 2017). A study by Racine et al. (2010) found that percentage of body fat

decreased in children who were overweight or obese following supplementation of CLA. Post-menopausal women showed a trend to increase in bone mineral density but no significant difference was observed when compared to the control therefore more research is required to assess the role of CLA in relation to bone mineral density (Brownbill et al., 2005).

1.3.6 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are mainly found in fish such as cod, halibut, mackerel, salmon and menhaden (Krumbeck et al., 2018). The PUFA α-linolenic acid is principally found in plants like seeds and nuts (Krumbeck et al., 2018). There are many types of PUFAs: α-linolenic acid (ALA), stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (Krumbeck et al., 2018). There is some controversy within the literature as to whether PUFAs can lower the risk of cardiovascular disease. A meta-analysis of 19 studies comprising of 45637 participants concluded that PUFAs are associated with reducing the incidence of fatal coronary heart disease with ALA, DPA and DHA having a moderately lower risk of fatal coronary heart disease (Del Gobbo et al., 2016). PUFAs have helped relieve depression with a recent meta-analysis consisting of 26 studies, finding that in particular EPA had a beneficial effect (Liao et al., 2019). Other health benefits are still being investigated, such as the impact PUFAs have on diabetic patients (Lalia and Lanza, 2016).

1.4 Probiotics

Probiotics have been in human use for over hundreds of years. Metchnikoff hypothesised that the consumption of lactic acid bacteria in fermented dairy improved the health of the Bulgarian labours (Anukam and Reid, 2007). The most widely used definition today is, ‘live micro-

organisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill et al., 2014). The following criteria are required to be classified as a probiotic, it must be able to be prepared on a large scale, remain stable during storage and use, survive the intestinal tract and the host must gain benefits from consuming the probiotic strain(s) (effects can either be a direct or an indirect via interaction with the existing microbiota) (Fuller, 1991, Scott et al., 2015). The endogenous microbiota may benefit from addition of a probiotic as it may be missing from the existing microbiota or it may help to stimulate existing species (Scott et al., 2015). Probiotics may compete for adhesion sites and nutrients with pathogenic microorganisms, they also can produce different antimicrobial compounds including organic acids thereby improving colonisation resistance/competitive exclusion (Schrezenmeir and Vrese, 2001).

Probiotics are strain specific and can differ due to enzyme activity, dose, formulation, number of times consumed in a day and the same species may vary in terms of how beneficial they are (Schrezenmeir and Vrese, 2001). Many health benefits have been found after consuming probiotics such as decrease in *Helicobacter pylori* infections, relief from Irritable Bowel Syndrome, improved mineral metabolism, lessening allergic reactions, reduced flatulence and lowering blood lipids (Schrezenmeir and Vrese, 2001).

1.4.1 Bifidobacterium

Bifidobacteria are an anaerobic Gram-positive rod-shaped bacteria which are non-motile, non-spore-forming and non-gas-producing bacteria (Turroni et al., 2009). They belong to the Actinobacteria phylum and are lactic acid producing bacterium (Candela et al., 2011).

Bifidobacterium spp. have been associated with an increase in organic acids like butyrate which helps colonocyte function (Louis and Flint, 2009). Bifidobacteria are unable to produce butyrate by fermenting substrates such as oligosaccharides but instead produce acetate

and lactate. Acetate can be converted by butyrate producing bacteria for example by *Faecalibacterium prausnitzii, Clostridium coccoides–Eubacterium rectale* group and *Roseburia* via mainly the butyryl-CoA : acetate CoA-transferase (Louis et al., 2010).

Interestingly, many patients with gastrointestinal syndromes and metabolic diseases have a decrease in *Bifidobacterium* in comparison with healthy volunteers (Tojo et al., 2014). The most prevalent species of *Bifidobacterium* in the human gut are *B. adolescentis, B. bifidum, B. breve, B. longum, B. angulatum, B. catenulatum, B. dentium, B. pseudocatenulatum* and *B. pseudolongum* although one of the most commonly used probiotics is *Bifidobacterium lactis* HN019 and BB-12 (Masco et al., 2005).

1.4.1.1 Bifidobacterium and the immune system

Interactions between the gut microbiota and host-immunity are important for maintaining homeostasis and preventing disease. Butyrate can have anti-inflammatory effects by upregulating interleukin-10 (IL-10) and producing regulatory T cells (Treg) as well as inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Meijer et al., 2010, Qiu et al., 2013). Additionally, *Bacteroides* spp. particularly *Bacteroides fragilis*, have found to secrete polysaccharide A which can increase the release of IL-10 (Mazmanian et al., 2005). Improved immune response from vaccination was found after administration of *B. longum* BB536 in 264 new-borns (Wu et al., 2016).

In early life, *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), *Bifidobacterium longum* subsp. *longum* (*B. longum*), *Bifidobacterium bifidum* (*B. bifidum*) and *Bifidobacterium breve* (*B. breve*) can be found in the infant gut - *B. infantis* and *B. bifidum* are important as they utilise human milk oligosaccharides (Turroni et al., 2011).

A large study involving 1755 new-borns (<34 weeks) showed that consuming *B. breve* M-16V significantly reduced necrotising enterocolitis, a condition that results in inflammation

of the intestine may lead to mortality (Patole et al., 2016). However, in another large study (1315 infants between 23 and 30 weeks) *B. breve* strain BBG-001 did not prevent necrotising enterocolitis and late-onset of sepsis in new-borns (Costeloe et al., 2016). More recently a systematic review consisting of nine studies observed that *B. breve* reduced the risk of necrotising enterocolitis whereas, *B. lactis* was found to have a greater impact (Hagen and Skelley, 2019).

The probiotic *Bifidobacterium longum* subsp. *infantis* was consumed by 24 infants with gastroschisis, which is when the bowel develops outside of the baby's body in the amniotic fluid during pregnancy, in pilot study reported an increase in numbers of bifidobacteria and lower clostridia but did not result in shorter hospital stay (Powell et al., 2016).

The consumption of *Bifidobacterium animalis* subsp. *lactis* BB-12 has been shown to reduce the risk of respiratory tract infection in 55 infants (1 month old) (Tan et al., 2017). The same strain investigated in 210 children, found no difference in numbers of gastrointestinal infections, respiratory tract infections, duration of symptoms or absence from day care after consuming the probiotic for 3 months at a dose of 1×10^9 CFU (Hojsak et al., 2016). However, the strain *B. longum* BB536 (5×10^9 CFU) was found to significantly reduce sore throat in Malaysian children (2-6 years old) (Lau et al., 2018).

Research outlined that *B. lactis* HN019 can have an impact on the immune response of elderly volunteers, which generally have a weakened immune response and are subject to developing diseases (Gill et al., 2000, Gill, 2001b, Gill, 2001a). A 3 week intervention of *B. lactis* HN019 increased helper T cells into peripheral circulation, improved cellular immune function, leukocyte phagocytosis, and tumoricidal activity thereby optimising and restoring immunity (Gill, 2001b).

In patients with *H. pylori* infection, probiotic *Bifidobacterium animalis* subsp. *lactis* B94 alongside eradication therapy resulted in a higher rate of eradication and less treatment for

diarrhoea and fewer side effects compared to the eradication therapy and placebo group and the eradication therapy only group (Çekin et al., 2016).

Celiac disease is an autoimmune disorder which is trigged by consuming gluten. A double-blind randomised, placebo-control trial in 49 children who have celiac disease found a reduction in TNF-α levels, a pro-inflammatory cytokine, after consuming *B. breve* for 3 months but the findings were reversed after intervention had ceased (Klemenak et al., 2015). A more recent study using the same probiotic in 40 coeliac children found that the probiotic increased *Actinobacteria* (Quagliariello et al., 2016).

1.4.1.2 The role of bifidobacteria in appetite regulation and blood glucose regulation

The gut microbiota may have a role in appetite regulation as a rodent study found that germ-free mice were leaner than conventional mice showing an increase in fat mass and insulin resistance (Backhed et al., 2004).

The groups *Clostridium* clusters IX and *Bacteroides* produce propionate which has been associated with increase in peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) hormones which regulate appetite and reduce energy expenditure (Cherbut et al., 1998, Tolhurst et al., 2012). This has been shown *in vivo* as infusion of propionate intra-colonic led to a significant increase in plasma concentration of PYY and GLP-1 (Psichas et al., 2015). After consuming an inulin-propionate ester, a significant increase in postprandial plasma PYY and GLP-1 occurred and after 24 weeks a reduction in weight gain was shown (Chambers et al., 2015).

Obesity is a global problem with approximately a third of the world's population being overweight or obese (Chooi et al., 2019). A randomised control trial in abdominally obese individuals found that *Bifidobacterium animalis* subsp. *lactis* Ba82145 at a dose of 1×10^{10} CFU for 3 months decreased waist circumference, body mass index and increased *Akkermansia*

spp. (Pedret et al., 2019). The genus *Akkermansia* has been associated with weight loss and may help reduce the risk of type 2 diabetes. *Bifidobacterium animalis* subsp. *lactis* B420 probiotic showed an increase in *Akkermansia* spp., and *Lactobacillus* spp. and the authors concluded that the probiotic may improve gut barrier function and obesity markers (Hibberd et al., 2019). A study involving 51 patients with metabolic syndrome, were randomly allocated to control group or *B. lactis* HN019 fermented in milk and found a positive impact on reducing BMI, total cholesterol, LDL and proinflammatory immune markers (TNFα and IL-6) (Bernini et al., 2016). In a study in severe obese volunteers assessing the role of probiotics (*Clostridium butyricum* MIYAIRI or *Bifidobacterium longum* BB536) or digestive enzymes after gastric bypass, all interventions showed improvements in gastrointestinal quality of life indices and symptoms such as excess gas, heartburn, belching and abdominal pain (Chen et al., 2015).

Additionally, gestational diabetes mellitus can often occur during pregnancy and in a study by Ebrahimi et al. (2019) found that the consumption of *B. lactis* HN019 for 8 weeks improved blood glucose control in pregnant women.

In summary, there is quite extensive research evidence for the role *Bifidobacterium* probiotics can have on reducing the risk of developing obesity.

1.4.1.3 The role of bifidobacteria on cognition

The gut-brain axis has recently received a lot of attention. The gut microbiota can directly signal to the brain through the vagal nerve or by releasing hormones into the blood flow. Interestingly, the body produces 90% of its serotonin, regulating mood, in the gut by synthesising enterochromaffin (EC) (Erspamer, 1954, Gershon and Tack, 2007). A high amount of serotonin has been associated with having a better mood whereas, low levels led to more depression. The most commonly used antidepressants inhibit the reuptake of serotonin relieving anxiety and depression. Having a high concentration of γ-aminobutyric acid (GABA),

an inhibitory neurotransmitter, has also been associated with improvements in anxiety and depression (Mazzoli and Pessione, 2016).

In vitro, Bifidobacterium and *Lactobacillus* strains have been shown to produce GABA when in the presence of the precursor monosodium glutamate (Barrett et al., 2012). This was supported by an *in vivo* study using a mouse model, as after consuming *Lactobacillus* an increase in GABA was expressed in the brain (Bravo et al., 2011). Additionally, *Bifidobacterium infantis* has been associated with antidepressant properties (Desbonnet et al., 2008).

In vivo cognitive tests are used to assess the impact of probiotics on memory and cognitive function. The probiotic *B. breve* A1 has been shown to be effective at improving immediate memory, delayed memory and mental state tests when comparing 117 elderly participants consuming either probiotic or placebo for 12 weeks (Kobayashi et al., 2019).

The strain *Bifidobacterium longum* 1714 has been shown to reduce cortisol therefore lowing the stress response. A randomised, double-blinded, placebo-controlled trial was carried out in 40 healthy participants who consumed *B. longum* 1714 ($1x\ 10^9$ CFU/day) for 4 weeks or participants consumed a placebo (2g maltodextrin) and response to a social stress test was observed (Wang et al., 2019). The outcome showed that the probiotic reduced response to the social stress stimuli (Wang et al., 2019). Similar findings were shown by Allen et al. (2016) where suppression of cortisol after social stress occurred, in addition, minor improvements in memory were found. The same strain has also been found to reduce depression in participants suffering from IBS but did not help to manage anxiety or improve quality of life (Pinto-Sanchez et al., 2017).

1.4.1.4 Bifidobacterium spp. and gastrointestinal diseases/disorders

Probiotics have been shown to help relieve and may result in clinical remission from certain gastrointestinal diseases. Ulcerative Colitis (UC) is characterised by chronic inflammation of the colon and rectum with many patients developing small colon ulcers; symptoms include diarrhoea, stomach pain and urgency to empty bowels. A dose of 2.5 x 10^{11} CFU of *B. longum* 536 for 8 weeks resulted in a reduction in UC disease index score and reduction in UC symptoms compared to a placebo group (Tamaki et al., 2016).

Irritable bowel syndrome (IBS) is classified through the Rome IV criteria. Diagnosis criteria includes recurrent abdominal pain which relates to defecation, change in frequency of stool or change in appearance of stool occurring once a week for at least 6 months (Hellstrom and Benno, 2019). A double-blind, randomised, placebo-control, parallel study was carried out in 275 volunteers who had abdominal pain and bloating (Ringel-Kulka et al., 2017). After consuming *B. longum* 35624, there was no significant difference in abdominal pain, bloating or the mean severity symptom score, although, there were more days which were bloat free (Ringel-Kulka et al., 2017).

1.4.1.5 The role of bifidobacteria and constipation

A common gastrointestinal complaint is constipation. In a systematic review, 11 RCT investigating constipation in children with IBS, functional abdominal pain disorder and functional constipation found insufficient evidence to recommend probiotics (Wegh et al., 2018). One probiotic to show positive benefits in reducing frequency and intensity of abdominal pain in children with IBS was *Lactobacillus rhamnosus* GG (Wegh et al., 2018). However, in a randomised control trial 65 participants found an increase in bowel movements after consuming 1 x 10^{9} or 1 x 10^{10} CFU of *Bifidobacterium animalis* subsp. *lactis* BB-12 for 28 days and the higher dose resulted in reduced straining (Ibarra et al., 2018). Similarly, in

1248 participants a randomised, double-blind, placebo-control trial found a significant increase in bowel frequency after the BB-12 intervention (1 x 10^9 CFU), although no additional benefits were found after consuming a high dose of 1 x 10^{10} CFU/day (Eskesen et al., 2015).

A study comparing fermented milk without probiotic and fermented milk with *Bifidobacteruim animalis* found no difference between conditions as both improved constipation symptoms (Moreira et al., 2017). Furthermore, *Bifidobacterium animalis* subsp. *lactis* NCC2818 found no effect on patients with chronic constipation after consumption for 4 weeks at a dose of 1.5 x 10^{10} CFU as there was no difference in transit time, stool output, symptoms and quality of life (Dimidi et al., 2018).

1.4.1.6 The role of bifidobacteria in diarrhoea

A large study in infants showed that in a probiotic *B. infantis* IM1 (1 x 10^7 CFU) group (73 participants) compared to control group (78 participants) a significant reduction in diarrhoea after 8 weeks and an association with lower risk of constipation (Escribano et al., 2018). Another strain which helps to relieve diarrhoea and soft stool was *B. infantis* EVC001 which was supplemented at a dose of 1.8-1.2 x 10^8 CFU with breast milk for 21 days (Smilowitz et al., 2017).

1.4.1.7 The role of bifidobacteria in Colic disease

Colic is the regular and prolonged crying in new-borns and can worsen at 4-6 weeks old. The use of *B. breve* B632 and BR03 was shown to significantly improve crying after 3 months of consumption at 1 x 10^8 CFU with an average crying time of 12.14 minutes in the probiotic group and 46.65 minutes in the placebo group (Giglione et al., 2016).

1.4.2 Lactobacillus

Lactobacillus spp. are Gram-positive, rod or coccobacilli shaped, non-spore forming, mostly facultative anaerobic bacteria (Wood and Holzapfel, 1995, Slover and Danziger, 2008). *Lactobacillus* belongs to the Firmicute phylum (Felis and Dellaglio, 2007). The genus makes up the largest proportion of the lactic acid bacteria (LAB) due to their main end product being lactic acid, additionally acetic and succinic acids are produced in smaller concentrations (De Angelis and Gobbetti, 2004, Slover and Danziger, 2008).

Lactobacillus spp. are starters for many food products such as cheese and yogurt and often found as probiotic supplements (De Angelis and Gobbetti, 2004). *Lacticaseibacillus casei* (formally known as *Lactobacillus casei*) strain Shirota by Yakult in 1935 was one of the first commercially available probiotic strains (Vasiljevic and Shah, 2008). Some of the most popular probiotic species used are *L. acidophilus*, *L. plantarum*, *L. casei*, *L. rhamnosus* and *L. reuteri* (Slover and Danziger, 2008).

1.4.2.1 Lactobacillus and the immune system

In preterm formula fed infants, feeding of 1 x 10^8 CFU of *Limosilactobacillus reuteri* DSM 17938 (used to be named *Lactobacillus reuteri, L. reuteri* DSM 17938) led to reduced number of days in hospital, less days on antibiotics, a shorter period of enteral feeding and an increase in anti-inflammatory IL-10. The placebo group was found to have increased pro-inflammatory IL-17, IL-8 and TNF-α (Indrio et al., 2017). This modulated the immune system however, another study did not show improvements in enteral feeding and growth measurements as the findings were similar to the placebo (*L. reuteri*, 1.25 x 10^8 CFU, studied in 134 low birthweight infants) (Wejryd et al., 2019). Additionally, no alterations in T cells were shown after consumption of *L. reuteri* in prenatal infants (Qazi et al., 2020).

Similar to *Bifidobacterium* species, the probiotic *L. rhamnosus* was shown to decrease the incidence of necrotising enterocolitis in 72 preterm infants compared to placebo (Singh et al., 2017). Also, the probiotic *L. reuteri* combined with high dose PPI- bismuth-containing quadruple therapy was effective at eradicating *H. pylori* in 100 infected patients (68% effectiveness after 7 days and 96% effectiveness after 14 days) and also lower nausea, vomiting, abdominal pain and bitter taste (Poonyam et al., 2019).

Lactobacillus strains have been widely researched in relation to allergies. *L. rhamnosus* GG combined with extensively hydrolysed casein formula was shown to limit the risk of developing functional gastrointestinal disorders in children cow's milk allergy (Nocerino et al., 2019). However, another study found that partially hydrolysed and extensively hydrolysed formulas could support the normal growth without the *L. rhamnosus* GG strain in a longitudinal study (5 years follow up) (Scalabrin et al., 2017). Six months of *L. rhamnosus* GG use in infants helped reduce the development of asthma and atopy but this was reversed 6 months after the probiotic supplement was stopped (Durack et al., 2018). This suggests that timing of intervention is critical as is duration of probiotic use.

Allergic rhinitis improved immunologic response following consumption of probiotic (*L. rhamanosus* GG) supplementation compared to vitamin D in children (Jerzynska et al., 2016).

Upper respiratory tract infection is an acute infection which frequently occurs in athletes following strenuous exercise therefore making them a good model to investigate the immune response. Modulation of the immune system was found in marathon runners, as higher anti-inflammatory and lower neutrophil infiltration was present post marathon following consumption of *L. casei* strain Shirota (4×10^{10} CFU) for 30 days prior (Vaisberg et al., 2019). Similarly, incidence of upper respiratory tract infection was monitored through a randomised control trial in 96 males and it was found that consuming *L. casei* strain Shirota significantly

lowered incidence, frequency and duration (Shida et al., 2017). Additionally, natural killer cells were inhibited and an increase in cortisol was shown (Shida et al., 2017). A study in highly active participants found that the same probiotic did not affect the incidence of upper respiratory tract infection but incidence was low in both groups. Although, the intervention did lead to a lower amount of antibody titres which represents a positive immune system (Gleeson et al., 2016). However, in endurance trained athletes consumption of *L. casei* for 7 days did not modify immune protection (Gill et al., 2016) nor showed any difference in cytokines and endotoxins (Gill et al., 2015).

In non-athletes, consumption of probiotic *Lactiplantibacillus plantarum* (previously called *Lactobacillus plantarum*) DR7 for 12 weeks decreased the duration of nasal symptoms and frequency of upper respiratory tract infection along with less pro-inflammatory cytokines in middle aged (30-60 years) participants and enhanced IL-10 and IL-4 in younger participants (<30 years) (Chong et al., 2019a). To investigate the viral load response in healthy adults, intranasal human rhinovirus was inoculated into 59 participants as well as consumption of probiotic *L. rhamnosus* GG or control drink. No significant difference between viral load in the control or probiotic group was found (Tapiovaara et al., 2016).

Consuming 1 x 10^{10} CFU of *L. rhamnosus* GG twice a day for 6 months by 196 elderly participants in nursing homes had lower occurrence of respiratory viral infection compared to placebo (Wang et al., 2018). Likewise, in a residential home, elderly participants (~85 years old) reduced their risk of infection, saw improved quality of life and bowel movement after consumption of *L. casei* strain Shirota for 6 months (Nagata et al., 2015). Solano-Aguilar et al. (2016) also found that *L. rhamnosus* GG could modulate the immune system in elderly participants measured through transcription levels.

1.4.2.2 The role of lactobacilli in metabolic syndrome

Metabolic syndrome is a cluster of conditions such as high blood pressure, overweight/obesity, high cholesterol levels and elevated blood sugar which can increase the risk of developing type 2 diabetes, stroke and heart disease. Probiotics have been shown to improve metabolic syndrome markers and lower the risk of developing more detrimental conditions. The probiotic *L. reuteri* V3401 found to improve metabolic syndrome when consumed for 12 weeks at a dose of 5 x 10^9 CFU (Tenorio-Jimenez et al., 2018). The probiotic *L. reuteri* V3401 (5 × 10^9 CFU) consumed for 12 weeks helped to lower IL-6 and soluble cell adhesion molecules which are activated when cells are damaged but did not lead to improvements in metabolic symptoms (Tenorio-Jimenez et al., 2019).

In a double-blind trial in 46 participants with type 2 diabetes, it was found that *L. reuteri* DSM 17938 (1 x 10^{10} CFU) for 12 weeks increased insulin sensitivity index and deoxycholic acid (secondary bile acid) (Mobini et al., 2017). The release of insulin was improved by an increase in glucose-stimulated GLP-1 and GLP-2 in the probiotic (*L. reuteri* SD5865) group compared to placebo (Simon et al., 2015). The strain *L. casei* 01 (1 x 10^8 CFU consumed for 8 weeks) was also found to improve 40 participants with type 2 diabetes as a decrease in fasting blood sugar, insulin concentration, insulin resistance and improvement in glycemic response was found (Khalili et al., 2019).

In mild hypercholesterolaemic participants who consumed *L. plantarum* ECGC 13110402 (2 x 10^9 CFU) it was found that reduced LDL, total cholesterol and increased HDL occurred (Costabile et al., 2017). However, *L. casei* did not alter lipid profiles after 8 weeks of consumption in participants with hyperlipidemia (Lee et al., 2017). Consuming the probiotic *L. casei* strain Shirota did not alter trimethylamine-N-oxide (TMAO), a gut derived metabolite associated with cardiovascular disease severity, in participants with metabolic syndrome after consuming the probiotic three times a day for 12 weeks (6.5 x 10^9 CFU) (Tripolt et al., 2015).

L. plantarum 299v has been shown to support vascular endothelial function and decrease systemic inflammation in 20 men with coronary artery disease (probiotic consumed for 6 weeks at a dose of 2 x 10^{10} CFU) (Malik et al., 2018).

1.4.2.3 The role of lactobacilli in cognitive function

Recently, research has been conducted on *Lactobacillus* probiotic strains and anxiety, stress and depression. A group of medical students were tested during examination period and it was found that 8 weeks of supplementation with *L. casei* strain Shirota has been claimed to relief of stress, gastrointestinal symptoms and increased gut microbial diversity (Kato-Kataoka et al., 2016). Similarly, Takada et al. (2016) conducted a comparably designed study and also found that the same strain supressed participants response to stress. Other probiotic strains *Lacticaseibacillus rhamnosus* (previously named *Lactobacillus rhamnosus*) HN001 and *L. plantarum* DR7 also showed a reduction in anxiety, stress and depression (Chong et al., 2019b, Slykerman et al., 2017). Additionally, *L. plantarum* has been associated with improvements in cognitive performance (Rudzki et al., 2019, Hwang et al., 2019).

However, not all results have shown to be positive, as in elderly participants (>65 years) there was no significant difference in well-being, stress or anxiety after a probiotic intervention (*L. reuteri*) (Ostlund-Lagerstrom et al., 2016). Likewise, in a cohort of 29 healthy males the probiotic *L. rhamnosus* JB-1 had no effect on mood, anxiety, stress, sleep or cognition parameters (memory, emotional recognition, attention and processing) (Kelly et al., 2017).

1.4.2.4 The role of lactobacilli in Irritable Bowel Syndrome

Intervention with the probiotic *L. acidophilus* NCFM did not lead to alteration in symptoms of IBS, quality of life, anxiety, depression, stool frequency and consistency between placebo and treatment after a 12 week consumption (Lyra et al., 2016). However, participants with severe

abdominal pain symptoms showed reductions with the probiotic intervention (Lyra et al., 2016). No significant improvement in IBS symptoms were found after consumption of *L. casei* strain Shirota for 8 weeks compared to placebo (Thijssen et al., 2016).

1.4.2.5 The role of lactobacilli in functional abdominal pain

Symptoms of functional abdominal pain (relieving frequency and intensity as well as reduced abdominal distension and bloating) were improved after supplementation of *L. reuteri* DSM 17938 for 4 weeks in children (6-15 years) but this did not alter overall attendance rates at school (Weizman et al., 2016). Similarly, in 55 children (4-18 years) probiotic intervention (*L. reuteri* DSM 17938, dose 1 x 10^8 CFU) led to more days without pain and a more prominent decrease in severity of symptoms (Jadresin et al., 2017). Additionally, stool type and duration of abdominal pain were unaltered by a probiotic (Jadresin et al., 2017).

1.4.2.6 The role of lactobacilli in constipation

Functional constipation in children showed no improvement after consuming *L. reuteri* DSM 17938 although in same strain in adults showed an improvement in gas production, abdominal discomfort, pain, bloating and helped defecation (Wegner et al., 2018, Riezzo et al., 2018).

1.4.2.7 The role of lactobacilli in diarrhoea

The use of probiotics containing a *Lactobacillus* strain to reduce incidence of diarrhoea in children is debatable, as many studies have found no difference in diarrhoea episodes. The strain *Limosilactobacillus reuteri* (previously known as *Lactobacillus reuteri*) DSM 17938 (2 x 10^8 CFU) in 247 children who were taking antibiotics, did not lower risk of antibiotic associated diarrhoea: occurrence of diarrhoea was 20% in the probiotic group and 13% in the placebo group (Kolodziej and Szajewska, 2019). The same finding was demonstrated in a study (involving 38 children) by Olek et al. (2017) using *L. plantarum* DSM9843.

Nosocomial diarrhoea (acute diarrhoea) in children found similar outcomes, as no significant difference was found in diarrhoea onset, duration, stool frequency and consistence after the probiotic intervention with various strains (*L. acidophilus* NCFM and *L. reuteri* DSM 17938) (Henryk and Hania, 2019, Urbańska et al., 2016, Hong Chau et al., 2018). A large cohort of 943 children (3 months to 4 years) with acute gastroenteritis was investigated in a prospective, randomised, double-blind trial and it was found that the strain *L. rhamnosus* GG (1×10^{10} CFU) showed no difference in duration of diarrhoea, vomiting, absence in day care or transmission rates when probiotics were consumed for 5 days (Schnadower et al., 2018).

A study in 66 children (2-60 months) from Botswana with acute diarrhoea, found that the probiotic *L. reuteri* DSM 17938 (5×10^{8} CFU) for 60 days reduced the recurrence of diarrhoea by 93% and increased the growth of the children (Pernica et al., 2017). Another study assessing the impact of probiotic (*L. rhamnosus* GG) and antibiotics or antibiotics and placebo on antibiotic-associated diarrhoea in 90 children found that the probiotic significantly reduced incidence, duration and complications compared to placebo (Esposito et al., 2018). Children (80 participants) who were provided with oral rehydration solution or oral rehydration solution with *L. rhamnosus* GG found that the frequency of stool defaecation was significantly lower with the probiotic (Ali, 2019). Similarly, probiotic use increased *Prevotella*, *Lactococcus*, *Ruminococcus* but decreased *Escherichia* plus, gastrointestinal issues and infections up to 3 years after the supplementation (Korpela et al., 2016).

There have been fewer studies assessing diarrhoea in adults though the study by Alberda et al. (2018) found positive results with the probiotic *L. casei* resulting in 12.5% of the intensive care group developing antibiotic associated diarrhoea whereas, the incidence was 31.3% in a placebo group. Additionally, only 1 person had *Clostridum difficile* infection compared to 3 in the placebo group (Alberda et al., 2018).

1.4.2.8 The role of lactobacilli in Colic disease

Colic has been studied in *Lactobacillus* strains. Savino et al. (2018b) found that crying was shorter and an increase in FOXP3, which helps to regulate T cells, was found along with an increase in faecal calprotectin (a marker of intestine inflammation) after 30 days supplementation (*L. reuteri* DSM 17938, dose 1 x 10^8 CFU) in 180 infants (Savino et al., 2018b). Likewise, comparing 25 healthy infants and 34 infants with colic showed that those on the probiotic (*L. reuteri* DSM 17938) for 28 days had significantly reduced crying, and an increase in FOXP3 (Savino et al., 2018a). The probiotic stain *L. rhamnosus* GG (4.5 x 10^9 CFU) for 4 weeks resulted in less daily crying compared to the placebo but the duration was the same (Partty et al., 2015). However, no difference in crying time after consumption of *L. reuteri* DSM 17938 in studies by Fatheree et al. (2017) and Nation et al. (2017) were reported and the strain *L. rhamnosus* GG also found no difference (Cabana et al., 2019).

Additionally, there have recently been multiple other topics of interest involving *Lactobacillus* probiotic such as improvements in osteoarthritis, fracture recovery, bacterial profiles with C-section delivery, lactose intolerance, kidney disease and iron absorption (Lei et al., 2016, Zhang et al., 2019, Garcia Rodenas et al., 2016, Hajare and Bekele, 2017, Pakdaman et al., 2016, Eidi et al., 2018, Hoppe et al., 2015, Rosen et al., 2019).

It is important to note that most of the clinical trials and RTC's mentioned above involve a single strain intervention but many probiotic studies use multiple strains to help achieve health benefits and positive outcomes by cross feeding of bacteria and building a support system for bacterial growth.

1.5 Synbiotics

The term synbiotic was first introduced in 1995 by Gibson and Roberfroid (1995) who defined it as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the

survival of live microbial dietary supplement … activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare.' Recently a new definition has been released which is more concise and allows further innovation of synbiotics (Swanson et al., 2020). This overarching definition is 'a mixture comprising live microorganisms and substrate(s) selectively utilised by host microorganisms that confers a health benefit on the host.' This is further broken down into two types of synbiotics: synergistic and complimentary. The complimentary synbiotic must consist of a prebiotic and probiotic and lead to one or more health benefits (Swanson et al., 2020). Whereas, a synergistic synbiotic is a substance which specifically enriches the health benefit of the live microorganism when administered together (Swanson et al., 2020). Below are a few examples of how synbiotics may play a role in improving host health.

1.5.1 Health benefits of synbiotics

1.5.1.1 Non-fatty liver disease

Studies have shown the added benefit of a synbiotic such as research looking into non-fatty liver disease, which is the build-up of fat in the liver. A 28 week intervention resulted in a significant decrease in alanine aminotransferase (indicator of liver damage) in the complementary synbiotic group (a mixture of probiotics with FOS) compared to placebo (Eslamparast et al., 2014). Likewise, the same synbiotic combination was found to lower fasting blood sugar, triglycerides, inflammation markers and fat build up in the liver to greater extent than the placebo (Mofidi et al., 2017). A well designed study by Javadi et al. (2018) investigated 67 non-fatty liver disease patients and found that high-sensitive C-reactive protein was significantly lower in the synbiotic group compared to the prebiotic alone, probiotic alone and placebo.

1.5.1.2 Overweight and obesity

A mixture of probiotics, FOS and vitamins were given to children who were overweight or obese and this was associated with a reduction in weight but the study design did not assess individual components of the synbiotic or include the vitamins as a control therefore it is difficult to conclude that the results were due to the synbiotic intervention (Kianifar et al., 2018, Safavi et al., 2013). However, Javid et al. (2020) found similar results when comparing the synbiotic (*Bacillus coagulans* GBI-30 and FOS) to a placebo, as improvements in fasting blood glucose, high sensitivity C-protein and total antioxidative capacity in 50 patients with type 1 diabetes. Another synbiotic (*L. acidophilus*, *L. rhamnosus*, *B. bifidum*, *B. longum*, *Enterococcus faecium* and FOS) resulted in weight loss, body mass index and anthropometrics although weight loss was not significant in the group of obese children (Ipar et al., 2015).

1.5.1.3 Synbiotics and IBS

Synbiotic treatment (*B. lactis* B94, 5 x 10^9 CFU and inulin, 900mg twice a day) has also shown to improve IBS symptoms such as bloating, constipation, mucus in faeces and belching. The synbiotic group also had a higher recovery rate (39.1%) compared to a prebiotic alone group (12.5%) (Bastürk et al., 2016). A synbiotic study involving consumption of *Bacillus coagulans* (15 x 10^7 spores) and FOS (100mg) in 23 IBS patients, showed further reduction in abdominal pain and diarrhoea frequency but had no effect on constipation (Rogha et al., 2014). Another synbiotic group (*L. plantarum*, *L. rhamnosus*, *L. gasseri*, *B. infantis*, *B. longum*, *L. acidophilus*, *L. salivarius*, *L. sporogenes*, *Streptococcus thermophilus*, inulin and tapioca-resistant starch) took synbiotics for 4 weeks and found improvements in flatulence although no improvements were found in subjective global satisfaction relief scores of IBS symptoms (Cappello et al., 2013).

1.5.1.4 Synbiotics and Helicobacter pylori

Helicobacter pylori infections can cause stomach ulcers and inflammation. Children aged 5 to 17 years were assigned to standard triple therapy (amoxicillin, clarithromycin and lansoprazole) half of the children (50 children) also received a synbiotic (*L. acidophilus, B. lactis, B. longum, B. breve, L. paracasei, L. plantarum, L. rhamnosus* and inulin) which increased eradication of *H. pylori* (88%) compared to the standard triple therapy alone (72%) (Sirvan et al., 2017). However, another study in children (6-16 years) investigating a different probiotic strain (*Bifidobacterium lactis* B94) with inulin, found no difference in *H. pylori* eradication or symptoms (Ustundag et al., 2017). A recent meta-analysis concluded that the role of synbiotics play in *H. pylori* eradication is still uncertain, therefore further research is required to show substantial evidence.

1.6 Micronutrients and their effect on the gut microbiome

Micronutrients are an important part of our everyday diet as the body is unable to produce many vitamins. However, some vitamins such as vitamin K and vitamin B are synthesised by the gut microbiota (Rowland et al., 2018).

Vitamin K is separated into two groups vitamin K1 and K2 and the gut microbiota can convert vitamin K1 into K2 (Imbrescia and Moszczynski, 2020). Vitamin K has been associated with blood coagulation, calcium metabolism and bone health (Tsugawa et al., 2012).

Vitamin B can be found in animal-based foods, leafy green vegetables, beans, and peas but there is limited bioavailability of vitamins due to reduction through cooking. Vitamin B can also be produced by gut bacteria as research has shown that riboflavin and biotin can be produced by *Bacteroides, Fusobacterium* and *Proteobacteria* (Magnusdottir et al., 2015). The phylum *Bacteroides* is responsible for producing the largest range of B-vitamins (Magnusdottir

et al., 2015). Vitamin B deficiency is associated with cognitive impairment, increase risk of developing cardiovascular disease, lower bone mineral density and an essential role in host immunity (Yoshii et al., 2019, Hin et al., 2006, Spence et al., 2005, Stone et al., 2004).

Vitamin D has anti-inflammatory properties, modulates the immune system and impacts on intestinal barrier integrity. In a recent meta-analysis of 12 RTC and 4 observational studies, vitamin D was shown to improve symptoms of IBD through the Harvey-Bradshaw index along with improvements in inflammation via reducing high sensitivity C-reactive proteins (Guzman-Prado et al., 2020).

Minerals are also important micronutrients, as previously mentioned the role prebiotics play in helping absorption of calcium and magnesium. The other main minerals which impact the gut microbiota are zinc and iron. Zinc impacts epithelial tight junctions as shown in Caco-2 cells as there were improvements in epithelial barrier function, reducing gut permeability (Wang et al., 2013). However, excess amounts of zinc have been associated with increased toxicity as *Clostridium difficile* can be enhanced by the presence of zinc (Zackular et al., 2016).

Iron is well-known for its role in red blood cell function and the majority of living organisms require iron (Beard et al., 1996). A study in children from Kenya found that after supplementation of iron there was a detrimental effect on the gut microbiome as an increase in pathogenic bacteria such as *Clostridium difficile, Clostridium perfringens* and *Escherichia coli* (Jaeggi et al., 2015). Similarly, in a study conducted in 139 African children (6-14 years) who were randomly assigned to an iron fortified biscuit or biscuit containing no iron for 6 months, it was found that an increase in enterobacteria occurred along with an increase in intestinal inflammation (Zimmermann et al., 2010). A large proportion of the effects of iron supplementation on the gut microbiota was conducted in anaemic and malnourished children, therefore often having pathogenic bacteria colonising their microbiota prior to the start of the study.

1.7 Conclusion

In conclusion, dietary supplements prebiotics, probiotics, synbiotics and micronutrients can modulate the gut microbiome which may lead to positive health outcomes. Some areas have a high amount of scientific evidence to support dietary intervention whereas, others require more robust methodology. Although, due to the fact that typically western diets do not contain adequate amounts of fibre and key micronutrients, there is a need to supplement with prebiotics, vitamins and minerals at adequate amounts. Specific supplementation of probiotics may needed for certain health outcomes as individual strains have different functional health benefits. It is important to note that in this review the scientific evidence examined single strain probiotic interventions whereas, a large amount of recent research combines multiple strains which may lead to difference health outcomes due to the cross feeding of bacteria.

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2 *Chapter 2:* Wheat dextrin, partially hydrolysed guar gum and inulin modulate bacterial metabolism in an *in vitro* anaerobic batch culture gut fermentation

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2.1 Abstract

Fermentation of the fibres wheat dextrin (WD) and partially hydrolysed guar gum (PHGG) may positively alter gut bacteria and lead to favourable metabolite production. The aim was to compare bacterial profiles after anaerobic fermentation of WD, PHGG and inulin. The anaerobic batch culture system had inulin (positive control), starch and blank (negative control) vessels as controls. Samples were collected for bacterial enumeration and organic acid production through fluorescent *in situ* hybridisation combined with flow cytometry and gas chromatography, respectively. Microbial populations fluctuated during the fermentations. Inulin significantly increased populations of *Bifidobacterium* spp., at all timepoints compared to baseline. The WD and PHGG vessels had higher counts of *Bacteroides* and *Clostridium* cluster IX at 8h in comparison to baseline counts but only the PHGG was found to be significantly different. Inulin and PHGG vessels had significantly higher amounts of butyrate compared to the starch vessels. Propionate production was greater in the WD and PHGG vessels compared to the inulin vessels. Propionate and butyrate are involved in maintaining immune homeostasis which is important in several disease states.

2.2 Introduction

The fibre content of western diets is lower (the majority of the UK population, 96% of women and 87% of met do not meet the recommended 30g/day of fibre intake) compared to non-western diets such as Ghanaians who on average consume 24.9g/day (Lie et al., 2018, NDNS., 2019). Consequently, increasing fibre consumption may have positive implications to health (Bindels et al., 2015). One feasible way to include more fibre in the diet is through prebiotics, which are substrates selectively utilised by host microorganisms conferring a health benefit (Gibson et al., 2017).

Inulin is the most studied and well-known prebiotic, recognised to selectively stimulate bifidobacteria (Gibson et al., 1995). Inulin can be found in chicory and Jerusalem artichoke as well as many other plants (Roberfroid, 2005). There are many examples of health benefits from consuming inulin such as relieving constipation and increasing stool frequency as shown following 4 weeks (12g per day) of inulin intake in 44 participants with chronic constipation as well as reducing serum lipopolysaccharide endotoxins, when consumed at doses of 13 g per day, found in 20 participants (Chu et al., 2019, Micka et al., 2017). The research in this area has led to an health claim by European Food Safety Authority (EFSA), “Chicory inulin contributes to maintenance of normal defecation by increasing stool frequency” (EFSA, 2015, Hond et al., 2000).

Two fibres which have been studied to a lesser extent, in this regard, are wheat dextrin (WD) and partially hydrolysed guar gum (PHGG).

The fibre wheat dextrin (WD) is a resistant dextrin containing non-digestible α-1,2 and α-1-3 linkages, it has a DP between 12-25 and average molecular weight of 4000-6000 Da (Noack et al., 2013). When consumed it is not fully digested in the small intestine and 76-87% reaches the colon where it is fermented (Van Den Heuvel et al., 2005, Vermorel et al., 2004).

Wheat dextrin has been shown to be tolerated at high doses, up to 45g per day, when used acutely and/or chronically (van den Heuvel et al., 2004, Pasman et al., 2006). Even though, this has been shown to be tolerable it would not be advised to consume this amount of WD to individuals who have GI issues.

The impact of WD on individuals with GI issues is less understood as the majority of the research has focused on overweight individuals and participants with type 2 diabetes. A study investigating women with type 2 diabetes found a significant decrease in lipopolysaccharide (endotoxin), proinflammatory immune markers (IFN-γ and IL-12) and a significant increase in anti-inflammatory immune markers (IL-10 and IL-4) and CD8 (important for T cell signalling) following 10g of wheat dextrin per day for 8 weeks (30 participants) compared to a maltodextrin control group consisting of 25 participants (Farhangi et al., 2018). Likewise, another study in patients with type 2 diabetes found that WD (10g per day for 8 weeks) led to a significant reduction in fasting insulin levels and immune markers (IL-6 and TNF-α) compared to the control group (maltodextrin) (Aliasgharzadeh et al., 2015).

Farhangi et al. (2018) also assessed the mental wellness and stress by measuring cortisol levels before and after WD consumption for 8 weeks and found a reduction in cortisol.

There have been inconsistent findings in the literature when assessing the impact of WD on the gut microbiome (Carlson et al., 2015, Gamage et al., 2018, Hobden et al., 2013, Noack et al., 2013).

Guar gum comes from the plant *Cyamoposis tetragonolobus* which is grown in India and Pakistan. Guar gum has a very high viscosity, therefore controlled partial enzymatic hydrolysis breaks down the linkages between the mannose backbone reducing viscosity and lowering molecular weight. The molecular weight of PHGG is between 1,000 and 100,000Da, with an average of 20,000Da, the structure of PHGG is similar to that of native guar gum as

linkages of the mannose backbone (β(1-4)- linkages) are broken down without altering the α(1-6) linked galactose units (Mudgil, 2018, Yoon et al., 2008).

The dietary fibre PHGG has been associated with improving GI problems such as constipation and diarrhoea (Dimitrios et al., 2014, Nakamura et al., 2007, Spapen et al., 2001).

Participants with chronic constipation (49 participants) consumed 5mg of PHGG for 4 weeks and found a reduction in transit time, an increase in frequency of bowel movements, reduced straining, decrease in abdominal pain and improvements in stool formation (Dimitrios et al., 2014).

In a model to induce diarrhoea, through the use of maltitol or lactitol, diarrhoea occurred in 85.3% to 100% of the 34 healthy volunteers and symptoms were relieved following 10g of PHGG (Nakamura et al., 2007). Spapen et al. (2001) studied patients suffering from sepsis and likely to develop diarrhoea through enteral feeding and showed that the addition of 22g/l of PHGG reduced the frequency and duration of diarrhoea compared to enteral feeding without added fibre.

The symptoms of diarrhoea and constipation are often observed in healthy individuals throughout their lifetime so it is important we understand how to relieve them and the impact different dietary fibre can have. Multiple studies using PHGG have been carried out in individuals who suffer from Irritable bowel syndrome (IBS) as this is a functional GI chronic disorder with a high prevalence (Dupont, 2014, Ringel et al., 2011, NICE, 2008). In 44 healthy volunteers who had a tendency towards IBS-D (predominantly diarrhoea) showed improvements in stool form following 3 months consuming 5g per day of PHGG. The gut microbiota was investigated and showed a significant increase in *Bifidobacterium* spp. compared to the control group (10g per day of maltodextrin) (Yasukawa et al., 2019). In IBS-C (predominantly constipation), participants showed significant improvements in stool consistency, form and improvements in symptoms, although improvements appeared to be

dependent on BMI, age and gender (Russo et al., 2015). Conversely, Niv et al. (2016) did not find any significant alteration in IBS symptoms or severity but an improvement in bloating and gas production following 12 weeks of consumption of 6g/d of PHGG (49 participants) compared to the control (59 participants).

A recent study on 188 IBS sufferers compared a fibre group (wheat bran 30g/day) to PHGG group (PHGG 5g/day) and found that both groups improved in IBS symptoms (bowel habits and abdominal pain) but PHGG was better tolerated (Parisi et al., 2002).

As such the dietary fibre are consumed in low amounts in the western diet and PHGG has been shown to reduce common GI symptoms such as diarrhoea, constipation and help relieve IBS associated symptoms. However, few studies have assessed the role the gut microbiome has on PHGG fermentation and bacterial metabolism (Carlson et al., 2016, Reider et al., 2020, Yasukawa et al., 2019). Therefore, this study will investigate and compare the bacterial metabolism of PHGG, inulin and WD. Bacterial populations will be measured by fluorescence *in situ* hybridisation combined with flow cytometry (FISH-FCM) and the production of organic acids analysed by gas chromatography (GC).

2.3 Material and Methods

2.3.1 Subjects

A total of three healthy volunteers (28±4 years old) donated samples. They had no history of gastrointestinal disorders and had not consumed antibiotics within the last 3 months or probiotics/prebiotics within the last 2 weeks.

2.3.2 Basal medium

The working volume of each vessel was 300ml and 135ml of autoclaved basal medium (peptone water 2g, yeast extract 2g, NaCl, 0.1g; K_2HPO_4 0.04g, $KHPO_4$ 0.04g, $MgSO_4.7H_20$ 0.01g, $CaCl_2.6H_20$, 0.01g $NaHCO_3$ 2g, Tween 80 2ml, haemin 0.05g, Vitamin K 10µl, L-cysteine 0.5g and bile salt 0.5g per litre) was incubated overnight with oxygen-free nitrogen sparging at a rate of 15ml/min.

2.3.3 Faecal sample

Participants brought faecal samples (samples were processed <3 hours from collection time) to the laboratory in an anaerobic jar (AnaeroJar™ 2.5 L, Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK) with a gas generation sachet (<1% O_2 and 9-13% CO_2, AnaeroGem™, Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK). The sample was diluted with phosphate buffered saline (PBS) 10% (w/v) (pH 7.4) and homogenised (Stomacher 400, Seward, West Sussex, UK) for 2 minutes at 240 paddle beats per minute. The vessels were inoculated with 15ml of faecal slurry.

2.3.4 Substrates

Substrates were added as follows: V1 blank, V2 WD (3.6g), V3 PHGG (3.6g), V4 inulin (3.6g) and V5 starch (0.54g). Wheat dextrin (Benefiber, GSK, Warren, New Jersey, USA) is a resistant dextrin containing non-digestible α-1,2 and α-1-3 linkages, it has a DP between 12-25 and average molecular weight 4000-6000 (Noack et al., 2013) (Figure 2.1). Inulin (Orafi®ST, Beneo, Mannheim, Baden-Württemberg, Germany) has an average DP of 12 (Roberfroid, 2005). PHGG (Resource Optifiber, Nestlé Health Science, London, UK) is made up of galactose, α-1-6 bonds, and mannose, β-1-4 bonds (Noack et al., 2013) (Figure 2.2).

Wheat dextrin is made up of 15% starch therefore a separate vessel with starch was used to determine whether alterations in gut bacteria were due to the presence of starch (Sigma Aldrich Ltd., Poole, Dorset, UK).

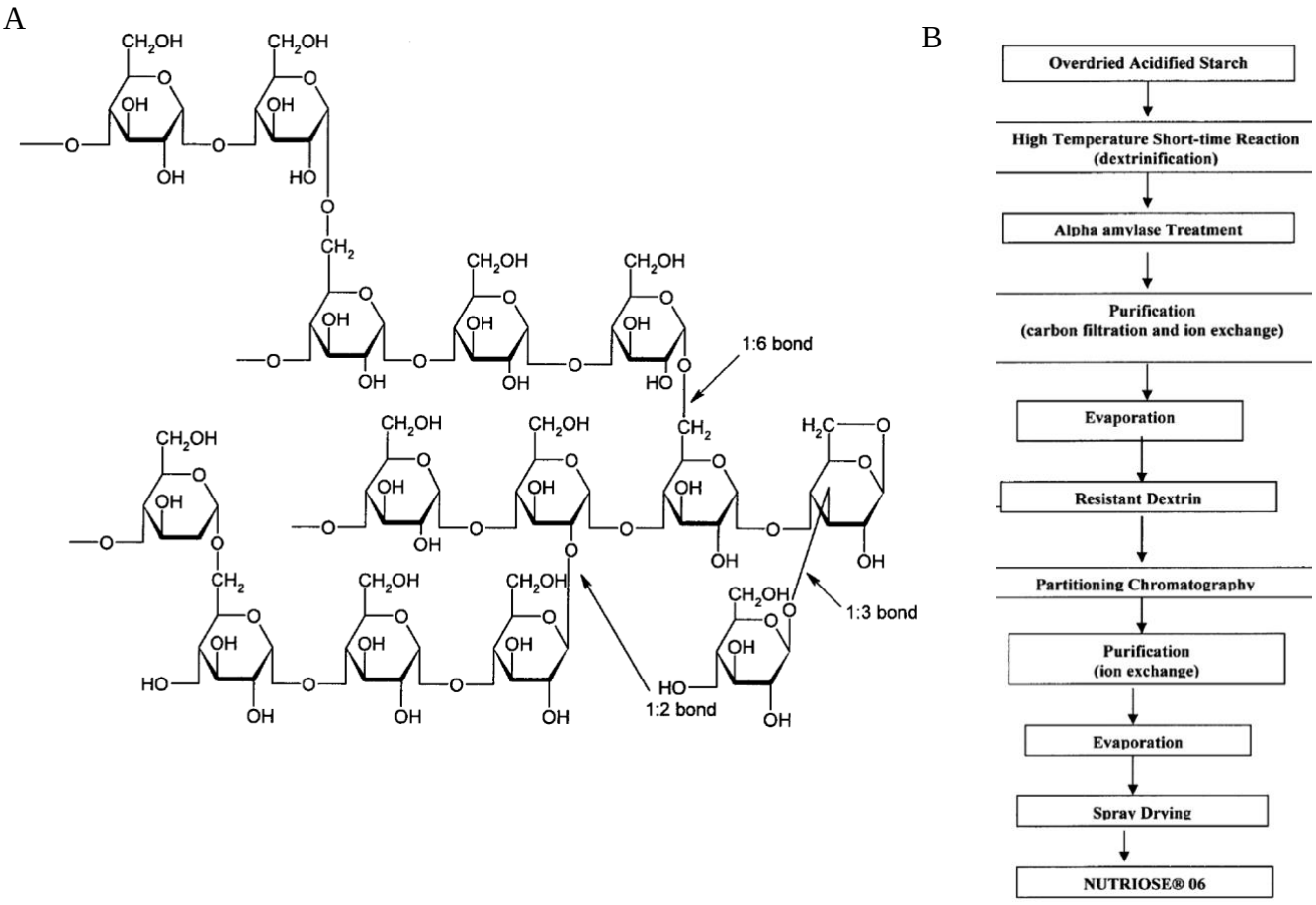

Figure 2.1. Structure and chemical composition of wheat dextrin (A) and the step by step process involved in producing wheat dextrin (B) (GRAS, 2012).

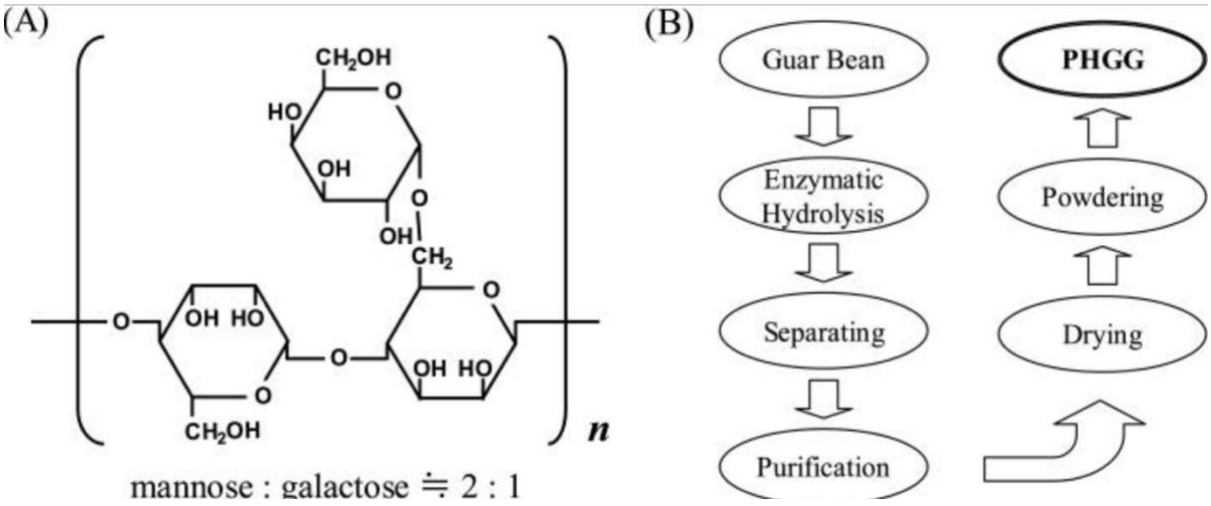

Figure 2.2. Chemical structure of partially hydrolysed guar gum (A) and the processed involved in producing partially hydrolysed guar gum (B) (Yoon et al., 2008).

2.3.5 Vessel conditions

Vessels were maintained at 37ºC via a circulating water bath and jackets around the fermenter and a controlled pH range between 6.7 to 6.9. The fermenters were continuously stirred throughout the experiment.

2.3.6 Samples

Samples were collected at 0, 8, 24, 48 and 72h. At each timepoint, 750µl was transferred to an Eppendorf tube and centrifuged at 11,337 × g for 5 minutes. The pellet was used for FISH-FCM and 500µl of supernatant for organic acid analysis via gas chromatography. The method used for FISH-FCM and organic acid analysis was previously reported in a study by Wang et al. (2019). The gut bacteria groups measured can be seen in Table 2.1.

Table 2.1. Bacterial probes names and DNA sequence used to detect common gut bacterial groups with validation references.

Probe name	Sequence (5' to 3')	Target group	References
Non Eub	ACTCCTACGGGAGGCAGC		(Wallner et al., 1993)
Eub338 I	GCT GCC TCC CGT AGG AGT	Most bacteria	(Daims et al., 1999)
Eub338 II	GCA GCC ACC CGT AGG TGT	Planctomycetales	(Daims et al., 1999)
Eub338 III	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	(Daims et al., 1999)
Bif164	CAT CCG GCA TTA CCA CCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*	(Langendijk et al., 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis;* all *Vagococcus, Pediococcus* and *Paralactobacillus* spp., *Melisococcus, Tetragenococcus, Catellicoccus, Enterococcus*	(Harmsen et al., 1999)
Bac303	CCA ATG TGG GGG ACC TT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz et al., 1996)
Erec482	GCT TCT TAG TCA RGT ACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb)	(Manz et al., 1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II)	(Franks et al., 1998)
Rrec584	TCA GAC TTG CCG YAC CGC	*Roseburia* subcluster	(Franks et al., 1998)
Prop853	ATT GCG TTA ACT CCG GCAC	Clostridial cluster IX	(Walker et al., 2005)
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium, Colinsella, Olsenella* and *Eggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium* and *Mycoplasma elephantis*	(Harmsen et al., 2000)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	(Hold et al., 2003)
DSV687	TAC GGA TTT CAC TCC T	Most Desulfovibrionales and many Desulfuromonales	(Devereux et al., 1992)

2.3.7 Statistical analysis

All statistics were performed in GraphPad Prism 7 (GraphPad Prism 7, San Diego, California, USA). FISH-FC and GC data were analysed using two-way mixed ANOVA to compare different test substrates and time points. Where significant differences were found, a post hoc analysis was performed using Tukey multiple comparison tests. Statistical analysis was accepted at $P < 0.05$ for all analyses.

2.4 Results

2.4.1 Bacterial enumeration

Total bacteria significantly increased by 1.1 $\log_{10}$ cells/mL in the inulin vessels ($P = 0.0032$) and by 0.93 $\log_{10}$ cells/mL in the PHGG vessels ($P = 0.0173$) at 8h and remained significantly higher until 24h compared to baseline, the inulin vessels were also found to be significant compared to the blank vessels (Figure 2.3A). *Bifidobacterium* spp. was significantly higher than baseline at all timepoints with an average increase of 1.65±0.16 $\log_{10}$ cells/mL ($P \leq 0.0290$) (Figure 2.3B). No increase in bifidobacteria were found in the PHGG and WD vessels. At 8h *Bacteroides* spp. significantly increased by 1.79 $\log_{10}$ cells/mL ($P = 0.0282$) in the inulin vessels and a greater significant increase, 2.09 $\log_{10}$ cells/mL, was found in the PHGG vessels ($P = 0.0071$) when compared to baseline (Figure 2.3C). The PHGG vessels contained a higher number of *Clostridium* cluster IX at 8h increasing by 1.48 $\log_{10}$ cells/mL when compared to baseline ($P = 0.0006$) (Figure 2.3D). A similar trend occurred in the WD vessels as the *Bacteroides* count was increased by 1.49 $\log_{10}$ cells/mL and *Clostridium* cluster IX increased by 0.74 $\log_{10}$ cells/mL at 8h but no significant differences were found.

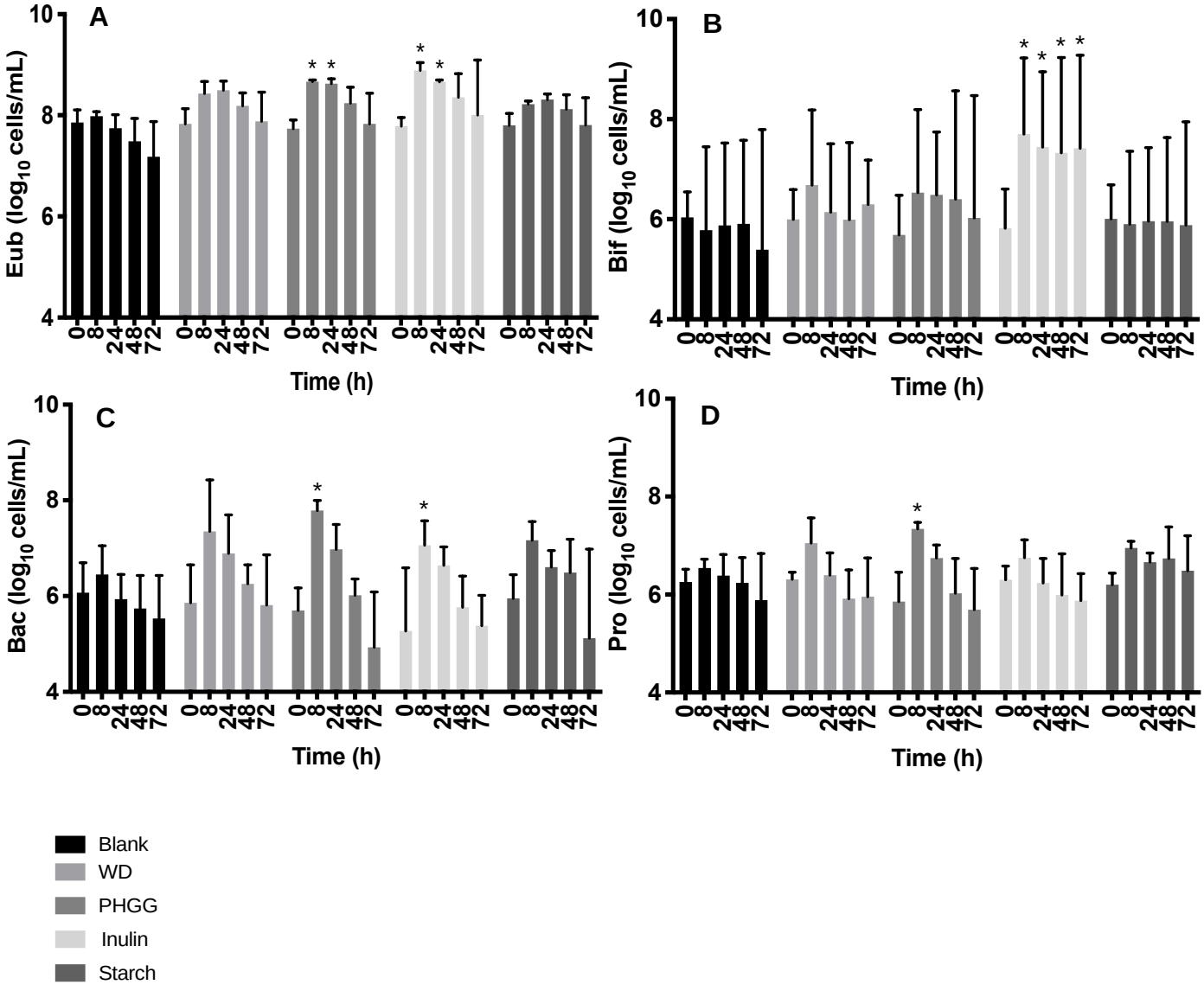

Figure 2.3. Bacterial enumeration from *in vitro* batch cultures on the fermentation profiles of blank (V1), wheat dextrin (V2), partially hydrolysed guar gum (V3), inulin (V4) and starch (V5) at 0, 8 24, 48 and 72 hours. The data are presented as mean ± SD (n=3) and * indicates significant difference ($P \leq 0.05$) compared to the baseline (T0). Bacteria measured were A = total bacteria (Eub) B = *Bifidobacterium spp.* (Bif), C = most Bacteroidaceae and Prevotellaceae (Bac) and D = *Clostridial* cluster IX (Pro).

2.4.2 Organic acid production

2.4.2.1 Acetate

Inulin significantly increased ($P < 0.0001$) the amount of acetate produced from baseline and compared to the blank vessels, the greatest increase was found at time point 24h, showing an increase of 118.08mM ($P < 0.0001$) (Figure 2.4A).

The PHGG containing vessels had an intermediate increase in acetate concentrations compared to the inulin and WD fermentations. A significant increase ($P < 0.0001$) in acetate concentrations occurred, at 24h there was a 113.88mM and 102.32mM increase, compared to the blank vessels and baseline concentrations, respectively. Additionally, acetate at 72h increased in the PHGG vessels by 69.55mM, $P = 0.0015$ compared to the same vessels at 8h.

At time point 8h, there was a significantly higher amount (57.58mM, $P = 0.0469$) of acetate in the inulin vessels compared to the WD vessels. This demonstrates that the WD vessels was slower to increase in acetate in comparison with inulin as significance was not found until 48h compared to the blank vessels, increasing by 66.64mM ($P = 0.0132$). However, there was a significantly higher amount of acetate in the WD vessels at 24h ($P = 0.0021$) compared to baseline.

2.4.2.2 Propionate

The WD and PHGG vessels both followed similar patterns, increasing concentrations of propionate in comparison to the blank vessels, increasing by 45.11mM at 48h ($P < 0.0001$) and 46.28mM at 72h ($P < 0.0001$), respectively (Figure 2.4C). Both vessels also showed a significant increase in propionate at 24h, 48h and 72h compared to time points 0h, 8h and the starch vessels.

The inulin vessels was significantly higher in propionate than baseline concentrations at 24h, the greatest increase was 26.26mM ($P = 0.0025$). Although, the PHGG containing

vessels had a greater concentration of propionate at 72h compare to the inulin vessels (41.41mM, $P = 0.0001$).

2.4.2.3 Butyrate

Intervention vessels followed a similar pattern with a significant increase ($P < 0.0001$) in butyrate from baseline (0h), 8h and the blank vessels (Figure 2.4B). The greatest increase was found in the inulin vessels at time point 48h with an increase of 47.67mM compared to baseline. Followed by the PHGG vessels which had an increase by 37.71mM at 72h and then the WD with the smallest increase, 26.24mM.

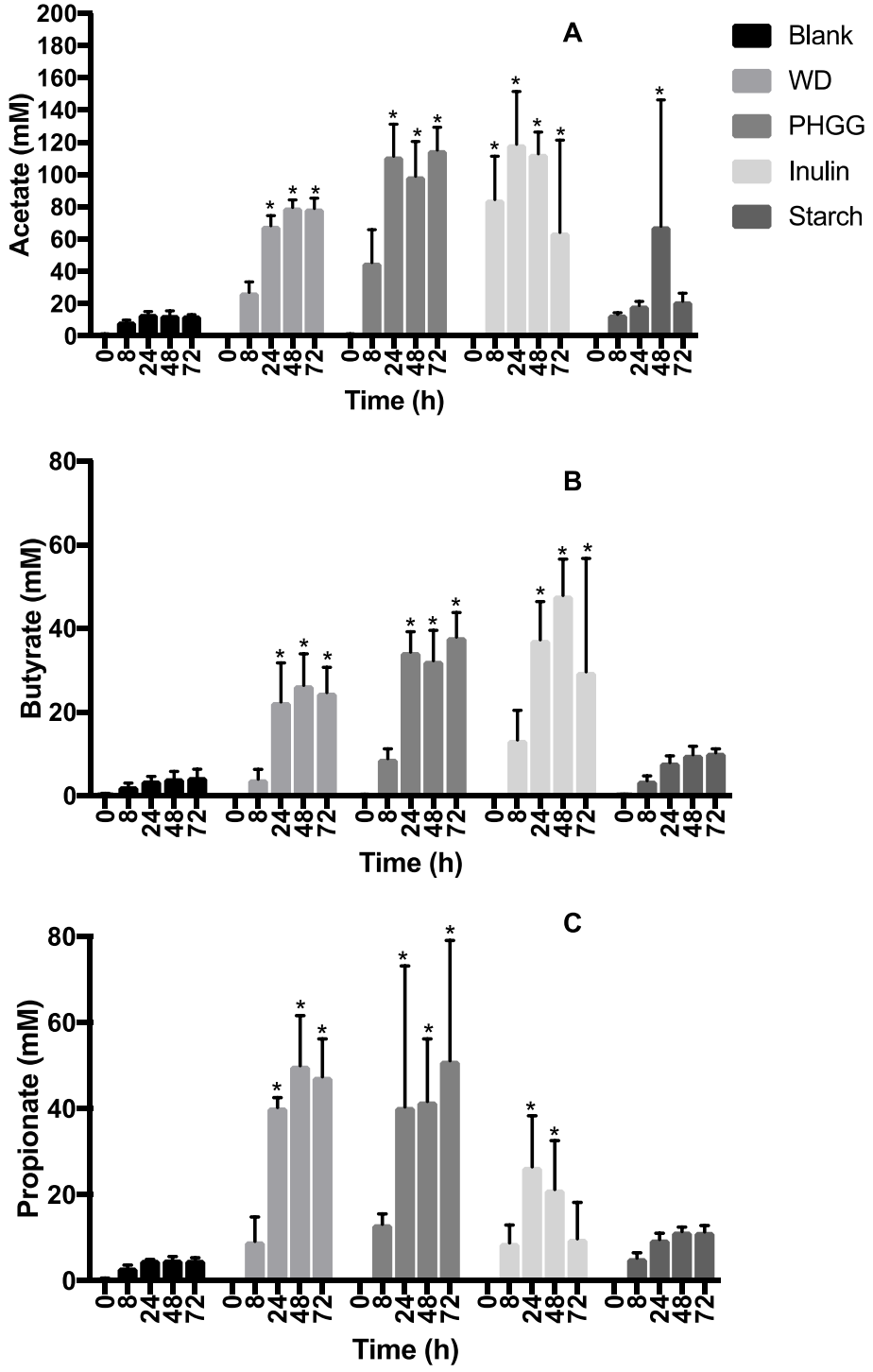

Figure 2.4.

Organic acid production from *in vitro* batch cultures on the fermentation profiles of blank (V1), wheat dextrin (V2), partially hydrolysed guar gum (V3), inulin (V4) and starch (V5) at 0, 8 24, 48 and 72 hours. The data are presented as mean ± SD (n=3) and * indicates significant difference ($P \leq 0.05$) from the baseline (T0). The organic acids measured were A = acetate, B = butyrate and C = propionate.

2.5 Discussion

The aim of this study was to compare gut bacterial profiles after faecal fermentation of WD, PHGG and inulin *in vitro.* The main findings were that PHGG significantly increased propionate, *Clostridium* cluster IX and *Bacteroides*. Similarly, in the WD vessel there was a significant increase in propionate production and an increase in *Bacteroides* and *Clostridium* cluster IX population from baseline but this was not significantly different.

There have been few studies investigating the effects of PHGG on the gut microbiota. A previous study by Reider et al. (2020) in healthy participants found an increasing abundance of *Ruminococcus, Fusicatenibacter, Faecalibacterium* and *Bacteroides* and a reduction in *Roseburia, Lachnospiracea and Blautia*. An *in vitro* culture study, fermenting samples in serum bottles, also showed an increase in *Parabacteriodes* and *Bacteroides* following inoculation with PHGG (Carlson et al., 2016). However, Yasukawa et al. (2019) showed, in participants who were healthy but had a tendency to IBS-D, an increasing abundance of *Bifidobacterium, Ruminococcus*, and *Megasphaera* and a reduction in *Bacteroides* and *Phascolarctobacterium*.

In this study, there was a significant increase in *Clostridium* cluster IX and *Bacteroides* in the PHGG vessel. Cluster IX contains many propionate producing bacteria for example *Veillonella, Megasphaera* and *Mitsuokella* (Walker et al., 2005). *Bacteroides* probes also contains *Prevotellaceae* and some *Porphyromonadaceae* which utilise the succinate-propionate pathway and studies have shown positive correlations between *Bacteroides* spp. and propionate production (Shimizu et al., 2018, Salonen et al., 2014). Short chain fatty acids (SCFA) are key fermentation end-points which have roles to play in providing energy for intestine epithelial cells, immunity, glucose metabolism, lipid metabolism, satiety and communication between the gut and the brain (He et al., 2020, Parada Venegas et al., 2019, Byrne et al., 2019). Usually, SCFA are absorbed in the colon (95%) therefore in many human

studies their detection in faeces is of limited value, although in the batch culture system, used in this study, SCFA accumulated in the vessels (Topping and Clifton, 2001). The most abundant SCFA was acetate, butyrate and propionate which were all significantly increased compared to baseline in all fibre interventions. The major difference was that propionate was found to be significantly higher in the WD and PHGG vessels compared to the inulin vessels. This is supported by previous *in vitro* studies (Noack et al., 2013, Stewart et al., 2009, Timm et al., 2010).

The SCFA butyrate and propionate have been shown to have an anti-inflammatory effect and play a role in the communicating between the microbiota and the immune system as they have the ability to inhibit histone deacetylase and regulate T cell production which is key to mediating the pro-inflammatory and anti-inflammatory system (Arpaia et al., 2013, Furusawa et al., 2013). They can also regulate the translocation of lipopolysaccharides (LPS) across tight junctions in the epithelium, butyrate has been shown to improve gut integrity by increasing tight junction proteins such as claudin-1 and zonula occludens-1 and reorganising occludin (Wang et al., 2012, Suzuki, 2013). Lipopolysaccharides are endotoxins which are produced by Gram-negative bacteria: if gut integrity is compromised, an influx of LPS from the gut to the blood stream can occur which may lead to inflammation (He et al., 2020, Salguero et al., 2019).

Patients with Inflammatory Bowel Disease (IBD), involving chronic inflammation of the gastrointestinal tract, often have lower concentration of SCFA compared to healthy volunteers (Lloyd-Price et al., 2019). Therefore, an *in vitro* study which used faecal samples from 10 Crohn's disease donors inoculated gut models with butyrate producing bacteria. Butyrate was significantly increased and improvements in epithelial barrier integrity were shown through a Caco-2 cell experiment (Geirnaert et al., 2017). Research has also shown a

positive impact of consuming sodium butyrate for 12 weeks in 66 patients with IBS helping to improve urgency, pain when defaecation and bowel habits (Banasiewicz et al., 2013).

In this study, no significant difference was found for *Bifidobacterium* spp. in the WD and PHGG vessels but there was a significant increase in the inulin vessels at all time points compared to baseline. Inulin has been widely found to be bifidogenic and therefore beneficial to the host (Reimer et al., 2020, Hiel et al., 2020, Kiewiet et al., 2020). Previous *in vitro* studies have also shown WD to lead to an increase in *Bifidobacterium* and *Lactobacillus* (Carlson et al., 2015, Noack et al., 2013). Although, a more recent study found a reduction in relative abundance of *Bifidobacteriaceae* (Gamage et al., 2018). Additionally, a study by Yasukawa et al. (2019) found a significant increase in abundance of *Bifidobacterium* following consumption of PHGG however, the subjects did have a tendency towards IBS-D symptoms. IBS sufferers have been shown to have a lower amount of *Bifidobacterium* (Kassinen et al., 2007, Malinen et al., 2005).

2.6 Conclusion

In conclusion, our findings showed that this study looked at bacterial enumeration following fermentation of WD, PHGG and inulin for 72h. The PHGG vessel significantly increased in *Bacteroides* and *Clostridium* cluster IX which are associated with higher propionate production (Shimizu et al., 2018, Salonen et al., 2014, Walker et al., 2005). This aligns with previous studies showing a significant increase in propionate in the WD and PHGG vessels compared to the inulin vessels. This may be of importance as propionate has anti-inflammatory effects which may be beneficial for individuals with GI issues. *Bifidobacterium* spp., which is well studied bacterium and has been associated with beneficial health outcomes, was significantly increased in the inulin vessels at all timepoints when compared to baseline. The WD and PHGG vessels did not significantly increase *Bifidobacterium* spp., therefore creating a synbiotic by

adding a probiotic may be a way to enhance beneficial effects on the host as well as increasing propionate production.

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3 Chapter 3: Metabolism of wheat dextrin, partially hydrolysed guar gum and inulin by *Bifidobacterium lactis* or *Lactobacillus acidophilus* in an *in vitro* gut model fermentation system

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Research Article

METABOLISM OF WHEAT DEXTRIN, PARTIALLY HYDROLYSED GUAR GUM AND INULIN BY *BIFIDOBACTERIUM LACTIS* OR *LACTOBACILLUS ACIDOPHILUS* IN AN *IN VITRO* GUT MODEL FERMENTATION SYSTEM

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3.1 ABSTRACT:

Combining the fibres wheat dextrin (WD), partially hydrolysed guar gum (PHGG) and inulin with a probiotic *Lactobacillus acidophilus* NCFM (NCFM) or *Bifidobacterium lactis* HN019 (HN019) may enhance bacterial metabolites leading to a healthier gut community. The aim of this study was to determine whether WD, PHGG and inulin or NCFM and HN019 alone generate a more favourable gut bacterial community than when combined. A secondary aim was to assess organic acid production following prebiotics, probiotics and synbiotic fermentation. An *in vitro* gut model batch culture fermentation was run for 72 hours. Samples were collected for bacterial enumeration (fluorescent *in-situ* hybridisation combined with flow cytometry) and organic acid production (gas chromatography). Inulin and HN019 combination

significantly increased bifidobacteria compared to inulin alone. Additionally, a significant increase in lactic acid bacteria, *Bacteroides* and *Clostridium coccoides–Eubacterium rectale* was found in the inulin containing probiotic vessels. The WD and PHGG vessels combined with the probiotic did not show any alteration in bacterial metabolism compared to the dietary fibres alone. In conclusion, synbiotic inulin combined with either HN019 or NCFM may help to enhance bacterial metabolites and cross feeding to lead to a prolonged elevation in *Bifidobacterium* spp., and lactic acid bacteria.

KEY WORDS: *Bifidobacterium lactis* HN019, Inulin, *Lactobacillus acidophilus* NCFM, Partially hydrolysed guar gum, Prebiotics, Probiotics, Synbiotics and Wheat dextrin.

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ABBREVIATIONS USED: ANOVA, Analysis of variance, $CaCl_2$, Calcium chloride, CFU/mL, colony forming units per millilitre, FISH-FCM, fluorescence *in situ* hybridisation flow-cytometry, HN019, *Bifidobacterium lactis* HN019, K_2HPO_4, Dipotassium hydrogen phosphate, KH_2PO_4, Monopotassium phosphate, $MgSO_4$, Magnesium sulfate, MRS, Man-Rogosa-Sharpe, NaCl, sodium chloride, $NaHCO_3$, Sodium bicarbonate, NCFM, *Lactobacillus acidophilus* NCFM, OD, optical density, PHGG, Partially hydrolysed guar gum, SPSS, Statistical package for the social and WD, wheat dextrin.

RUNNING TITLE: **Bacterial metabolism of synbitoics**

3.2 INTRODUCTION

Alterations in the gut microbiota have been associated with a range of disease states. Research has identified that medication, infection, poor diet, lifestyle and aging can result in a range of pathologies such as obesity, diabetes, inflammatory bowel disease, gastroenteritis and possibly digestive cancers (Gagliardi *et al.,* 2018; Hou *et al.,* 2011; Qin *et al.,* 2012; Turnbaugh *et al.,* 2006). The majority of bacteria in the human body reside in the colon with 10^{12} bacteria/g and

dietary intervention is the predominant method used to alter the intestinal ecosystem (Sender *et al.,* 2016).

One well-known intervention is a prebiotic, which is a 'substrate that is selectively utilised by host microorganisms conferring a health benefit' (Gibson *et al.,* 2017). An established prebiotic is inulin which is known for being bifidogenic leading to certain health benefits, for example improving cardiometabolic inflammation and increasing fat oxidation (Nicola *et al.,* 2018; Van der Beek *et al.,* 2018). Inulin is found in chicory and Jerusalem artichoke and comprises of fructose joined by ß-(2-1) linkages (Roberfroid, 2015). Another fermentable dietary fibre is wheat dextrin (WD) which is resistant dextrin made up of non-digestible α-1,2 and α-1-3 linkages. In the diet, it is not fully digested and 76-87% reaches the colon (Van Den Heuvel *et al.,* 2005; Vermorel *et al.,* 2004). Partially hydrolysed guar gum (PHGG) is a dietary fibre made from controlled hydrolysis of Guar Gum.

Previous research including the study reported in Pyle *et al.,* (2020, unpublished) found that WD and PHGG significantly stimulated *Bacteroides* and *Clostridium* cluster IX which led to an increase in propionate production. Propionate has been associated with appetite regulation and lowering of energy intake (Chambers *et al.,* 2015). Unlike inulin, WD and PHGG do not increase levels of positive groups like bifidobacteria or lactobacilli which have extensive health benefits including in human trials lowering cholesterol in hypercholesterolaemia participants, an effective treatment for acute diarrhoea in children when combined with oral rehydration therapy, improved in immune response in elderly volunteers and able to produce neurotransmitter γ-aminobutyric acid in the gut (Barrett *et al.,* 2012; Gill, 2001; Jones *et al.,* 2012; Simakachorn *et al.,* 2000). The addition of live bifidobacteria or lactobacilli combined with a prebiotic may lead to further health benefits to the host, this is termed a synbiotic. Synbiotics work in two ways either complementary or synergistically. The probiotic is specific strain(s) of bacteria with health benefits and a prebiotic either increases

indigenous microbiota compounds or helps survival of the probiotic (Kolida and Gibson, 2011).

Synbiotics were first considered by Gibson and Roberfroid (1995). Fewer papers have been published on synbiotics in comparison to prebiotic and probiotics but have increased in popularity with over 140 papers published on synbiotics in 2016 (Krumbeck et al., 2018). The majority of these papers are in rodents or *in vitro*. The few papers conducted in humans mainly found improvements in post operation infection, lower development of sepsis, reduced stay in hospital and shorter duration of antibiotic use (Kinross *et al.,* 2013; Sawas *et al.,* 2015).

A reduction in inflammation can be beneficial for many disease states such as ulcerative colitis which is chronic inflammation of the gastrointestinal tract. Furrie *et al.* (2005) found that ulcerative colitis patients had a reduction in inflammation markers such as tumor necrosis factor alpha and interleukin 1 alpha and that epithelial tissue started to regenerate after consumption of Synergy1 (6g) and *Lactobacillus longum* (2 x 10^{11} CFU/mL). Metabolic diseases may benefit from consumption of synbiotics with fructo-oligosaccharides (10g) and *Lactobacillus salivarius* (2 x 10^{9} CFU/mL) reducing inflammation, total cholesterol and low-density lipoproteins (Rajkumar *et al.,* 2015). There is also some evidence that a mixture of probiotics (*Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis*) and inulin can diminish diarrhoea (Ringel-Kulka *et al.,* 2015). The majority of these human studies lack adequate controls which lead to inconclusive data on whether the synbiotic is more beneficial than the prebiotic or probiotic alone. Additionally, most of these human studies did not assess the gut microbiota communities after the intervention.

Therefore, the aim of the study is to determine whether the carbohydrates WD, PHGG and inulin or probiotics *Lactobacillus acidophilus* and *Bifidobacterium lactis* alone

generate a more favourable gut bacteria community than when combined. A secondary aim was to assess organic acid production from the prebiotics and probiotics alone and combined.

3.3 MATERIALS AND METHODS

3.3.1 Subjects

Three healthy volunteers (30±2 years old) donated faecal samples. The volunteers had no history of gastrointestinal disorders and had not consumed antibiotics in the last 3 months or prebiotic/probiotic in the last 2 weeks. Ethical approval was obtained from University of Reading research ethics committee.

3.3.2 Faecal sample and incubation protocols

Participants brought a fresh faecal sample (<3 hours) to the laboratory in anaerobic conditions (<1% O_2 and 9-13% CO_2) (AnaeroJarTM 2.5 L and AnaeroGemTM, Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK) the next morning. The sample was diluted with phosphate buffer saline (PBS) 10% (w/v) (pH 7.4) and homogenised (Stomacher 400, Seward, West Sussex, UK) for 2 minutes at 240 paddle beats per minute. Twelve vessels were inoculated with 15ml of faecal slurry with a total working volume of 300ml. These vessels were prepared as follows. Each vessel received 135ml autoclaved basal medium (Peptone water 2g, yeast extract 2g, NaCl 0.1g, K_2HPO_4 0.04g, KH_2PO_4 0.04g, $MgSO_4.7H_20$ 0.01g, $CaCl_2.6H_20$ 0.01g, $NaHCO_3$ 2g, Tween 80 2ml, haemin 0.05g, Vitamin K 10µl, L-cysteine HCL 0.5g and bile salt 0.5g per litre) (Sigma, St. Louis, MO), prebiotics and probiotic bacteria as outlined below and incubated overnight in anaerobic conditions (oxygen-free nitrogen at a rate of 15ml/min).

V1- Blank,
V2- Wheat dextrin (3.6g),
V3- Partially hydrolysed guar gum (3.6g),
V4- Inulin (3.6g),
V5- *B. lactis* HN019 ($6x10^7$ CFU/mL),
V6- *L. acidophilus* NCFM ($5x10^7$ CFU/mL),
V7- Wheat dextrin and *B. lactis* HN019 ($6x10^7$ CFU/mL),
V8- Partially hydrolysed guar gum and *B. lactis* HN019 ($6x10^7$ CFU/mL),
V9- Inulin and *B. lactis* HN019 ($6x10^7$ CFU/mL),
V10- Wheat dextrin and *L. acidophilus* NCFM ($5x10^7$ CFU/mL),
V11- Partially hydrolysed guar gum and *L. acidophilus* NCFM ($5x10^7$ CFU/mL),
V12- Inulin and *L. acidophilus* NCFM ($5x10^7$ CFU/mL)

Wheat dextrin (Benefiber, GSK, Warren, New Jersey, USA) is a resistant dextrin containing non-digestible α-1,2 and α-1-3 linkages with a degree of polymerization between 12-25 and average molecular weight 4000-6000 Da (Noack *et al.*, 2013). Partially hydrolysed guar gum (Resource Optifiber, Nestlé Health Science, London, UK) comprises of galactose (α-1-6 bonds) and mannose units (β-1-4 bonds) (Noack *et al.*, 2013). Inulin has an average degree of polymerization of 12 and is made up of fructose joined by ß-(2-1) linkages (Roberfroid, 2005).

The probiotic *Bifidobacterium lactis* HN019 (Danisco Brazil, Cotia) is anaerobic, Gram-positive, non-spore forming bacteria which are safe for human consumption (GRAS, 2012). The probiotic *Lactobacillus acidophilus* NCFM (Danisco Brazil, Cotia) is a rod-shaped, non-motile, non-spore forming lactic acid bacterium. *Lactobacillus* is safe to consume and is found in a range of dairy products (GRAS, 2010). The HN019 and NCFM strains were freeze dried and stored at -4°C prior to use. The strains were grown in anaerobic conditions at 37°C in de Man-Rogosa-Sharpe (MRS) (Oxoid Ltd, Basingstoke, Hampshire, UK) broth and de Man-Rogosa-Sharpe supplemented with 0.05% (w/v) cysteine (MRS-C, Sigma, St. Louis, MO), respectively. The probiotics were grown at different dilutions in triplicate for 24h to produce a growth curve of optical density (OD_{600}) against colony forming units per millilitre (CFU/mL) to determine a dilution factor for the batch culture experiment to obtain $5x10^7 \pm 1x10^7$ CFU/mL (Figure 3). One day prior to inoculating the vessels, strains were

grown in the above conditions for 24h then on the experimental day diluted with PBS according to the growth curve dilution factor and immediately added to the vessels.

3.3.3 Incubation conditions and sampling

The vessels were maintained at body temperature (37ºC) through a circulating water bath and jacket around the fermenters while maintaining a pH between 6.7-6.9. They were continuously stirred throughout the experiment.

A 750µl sample was collected from each vessel after 0, 8, 24, 48 and 72 hours. The samples were centrifuged at 11,337 X g for 5 minutes. The resulting pellet was used for bacterial enumeration through fluorescence *in situ* hybridisation flow-cytometry (FISH-FCM) and 500µl of supernatant used to assess organic acid production via gas chromatography. The method used to analyse FISH-FCM and organic acid production are reported in detail elsewhere Wang *et al.* (2019). Bacterial groups measured within the study can be seen in Table 3.1.

Table 3.1. Bacterial probes names and DNA sequence used to detect common gut bacterial groups with validation references.

Probe name	Sequence (5' to 3')	Target group	References
Non Eub	ACTCCTACGGGAGGCAGC		Wallner *et al.,* (1993)
Eub338 I	GCT GCC TCC CGT AGG AGT	Most bacteria	Daims *et al.,* (1999)
Eub338 II	GCA GCC ACC CGT AGG TGT	Planctomycetales	Daims *et al.,* (1999)
Eub338 III	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	Daims *et al.,* (1999)
Bif164	CAT CCG GCA TTA CCA CCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*	Langendijk *et al.,* (1995)
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis;* all *Vagococcus, Pediococcus* and *Paralactobacillus* spp., *Melisococcus, Tetragenococcus, Catellicoccus, Enterococcus*	Harmsen *et al.,* (1999)
Bac303	CCA ATG TGG GGG ACC TT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	Manz *et al.,* (1996)
Erec482	GCT TCT TAG TCA RGT ACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb)	Manz *et al.,* (1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II)	Franks *et al.,* (1998)
Rrec584	TCA GAC TTG CCG YAC CGC	*Roseburia* subcluster	Franks *et al.,* (1998)
Prop853	ATT GCG TTA ACT CCG GCAC	Clostridial cluster IX	Walker *et al.,* (2005)
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium, Colinsella, Olsenella* and *Eggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium* and *Mycoplasma elephantis*	Harmsen *et al.,* (2000)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	Hold *et al.,* 2003
DSV687	TAC GGA TTT CAC TCC T	Most Desulfovibrionales and many Desulfuromonales	Devereux *et al.,* (1992)

3.3.4 Statistical analyses

All statistical analyses used SPSS version 25 (SPSS Inc, Chicago, Ill, USA). The FISH-FCM and organic acid production were analysed using two-way mixed ANOVA to compare different test substrates and time points. Where significant differences were found, a post hoc analysis was performed using Tukey multiple comparison tests. A paired t-test was used to further analyse the difference between vessels at each time point. Statistical analysis was accepted at $P < 0.05$ for all analyses.

3.4 RESULTS

3.4.1 Effect of wheat dextrin in combination with *B. lactis* HN019 and *L. acidophilus* NCFM on bacterial growth

The WD vessels significantly increased total bacteria by 0.91 $\log_{10}$ at 24h ($P = 0.046$). There was also a significant increase in lactic acid bacteria, (0.42 $\log_{10}$ cells/mL increase, $P = 0.046$), *Bacteroides* (0.9 $\log_{10}$ cells/mL increase, $P \leq 0.035$), *Clostridium coccoides–Eubacterium rectale* (0.9 $\log_{10}$ cells/mL increase, $P \leq 0.035$), *Roseburia* (0.7 $\log_{10}$ cells/mL increase $P = 0.001$) and *Clostridium* cluster IX (1.7 $\log_{10}$ cells/mL increase $P = 0.035$) (Figure 3.1A). The vessels with HN019 and WD generated higher *Bacteroides* (0.78 $\log_{10}$ cells/mL increase $P = 0.024$) compared to the WD vessels. This was elevated further in the NCFM and WD vessels with an increase of 1.86 $\log_{10}$ cells/mL ($P = 0.010$). Additionally, in the NCFM and WD vessels there was a significant increase in *Clostridium coccoides–Eubacterium rectale* (1.03 $\log_{10}$ cells/mL increase $P \leq 0.038$) which increased more rapidly in the first 8h than the WD vessels. The count of *Clostridium* cluster IX (1.39 $\log_{10}$ cells/mL increased $P = 0.049$) significantly increased in the NCFM and WD vessels but at an overall lower count than the WD vessels.

3.4.2 Effect of partially hydrolysed guar gum in combination with *B. lactis* HN019 and *L. acidophilus* NCFM on bacterial growth

PHGG significantly increased total bacteria at 8 and 24h ($P \leq 0.047$), *Bifidobacterium* spp., at 24 and 72h ($P \leq 0.032$), *Bacteroides* at 24h ($P = 0.08$), *Clostridium coccoides–Eubacterium rectale* at 48h ($P = 0.048$), *Atopobium* spp., *at* 48h ($P = 0.022$) and *Clostridium* cluster IX at 8h ($P = 0.034$) (Figure 3.1B). The HN019 and WD vessels significantly increased at 24h ($P = 0.044$) at a similar amount to the PHGG vessels. HN019 and WD vessels also increased in *Bacteroides* ($P = 0.04$) but at a quicker rate (8h) and higher count (0.3 $\log_{10}$ cells/mL higher)

than the PHGG vessels. The NCFM and PHGG vessels significantly increased *Bifidobacterium* spp., at 8h (P = 0.017) 0.26 $\log_{10}$ cells/mL more than the PHGG vessels. *Atopobium* spp., was significantly elevated in the NCFM and PHGG vessels ($P \leq 0.27$) 0.73 $\log_{10}$ cells/mL higher than the PHGG vessels.

3.4.3 Effect of inulin in combination with *B. lactis* HN019 and *L. acidophilus* NCFM on bacterial growth

There was a significant increase in *Bifidobacterium* spp., ($P \leq 0.035$) and *Atopobium* spp., ($P \leq 0.015$) in the inulin containing vessels (Figure 3.1C). The HN019 and inulin vessels had a significantly higher count of *Bifidobacterium* spp., ($P \leq 0.039$) with 24h having a 1.76 $\log_{10}$ cells/mL increase. The HN019 and inulin vessels had a 0.23 $\log_{10}$ cells/mL higher amount of *Bifidobacterium* spp., than the inulin vessels alone. The lactic acid bacteria count significantly increased in the HN019 and inulin vessels (P = 0.049) and also in the NCFM and inulin vessels (P = 0.017). The NCFM and inulin vessels also increased total bacteria (P = 0.018), *Bacteroides* ($P \leq 0.015$), *Clostridium coccoides–Eubacterium rectale* (P = 0.026) and *Atopobium* spp., (P = 0.022).

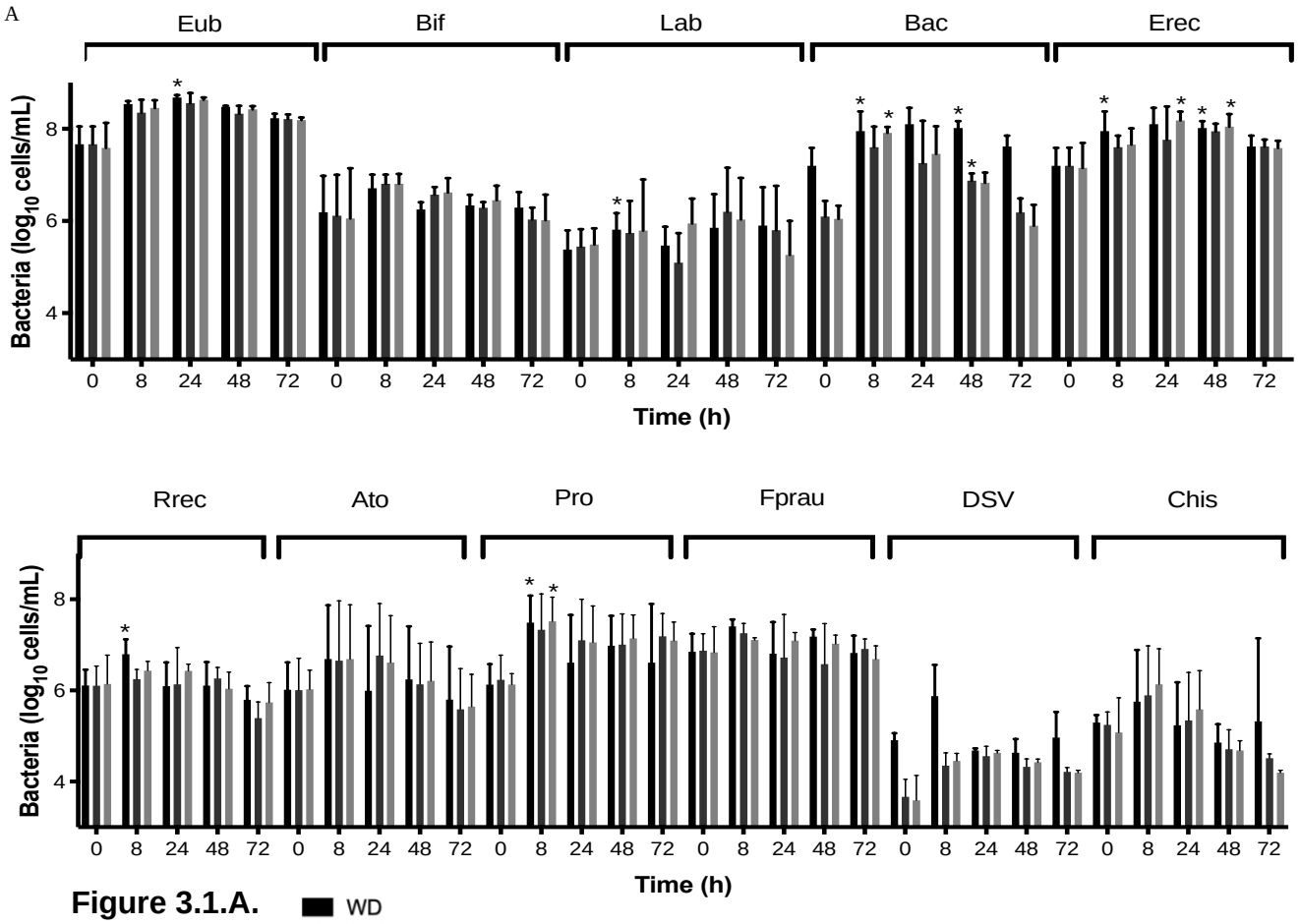

Figure 3.1.A.

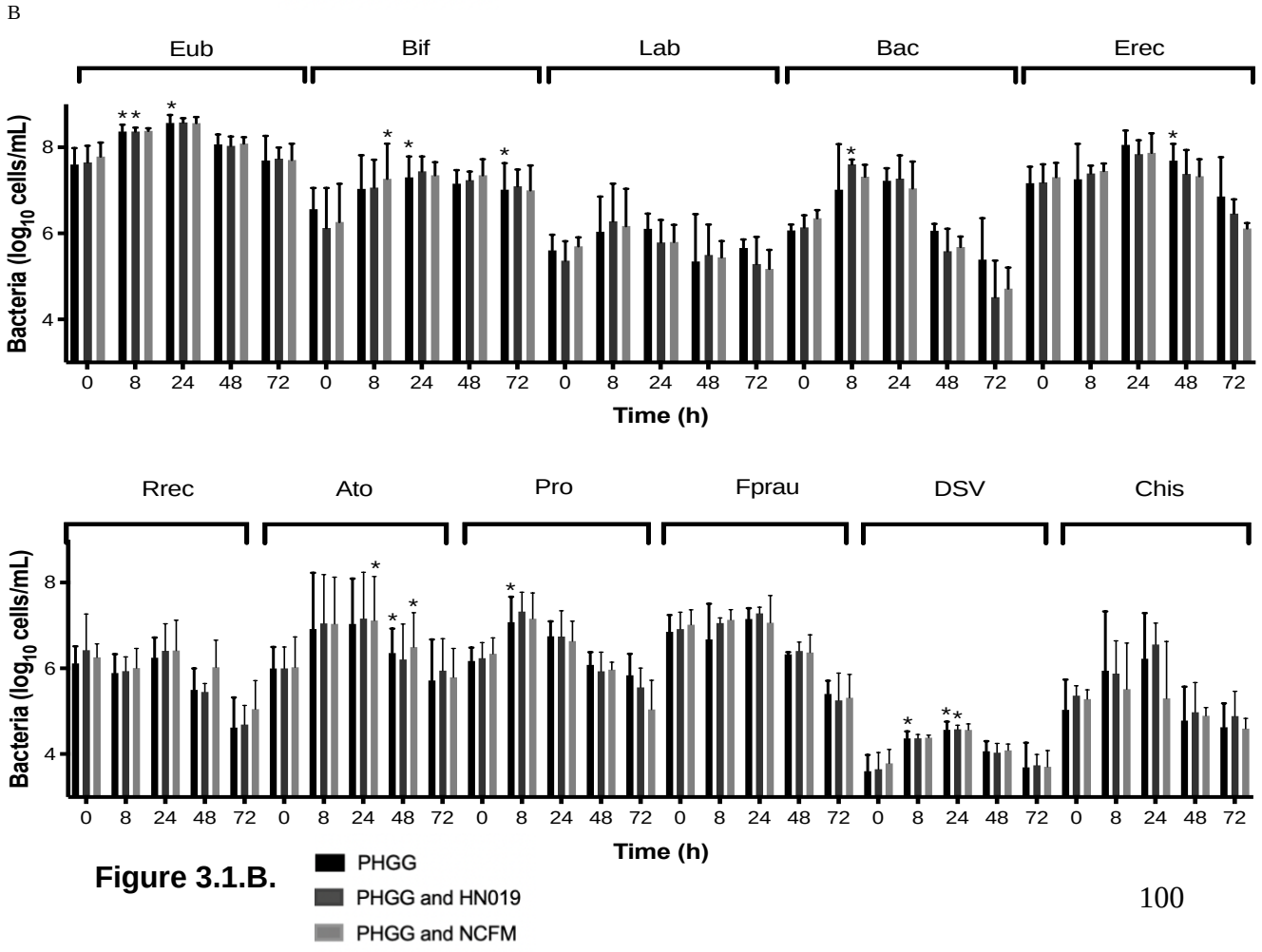

Figure 3.1.B.

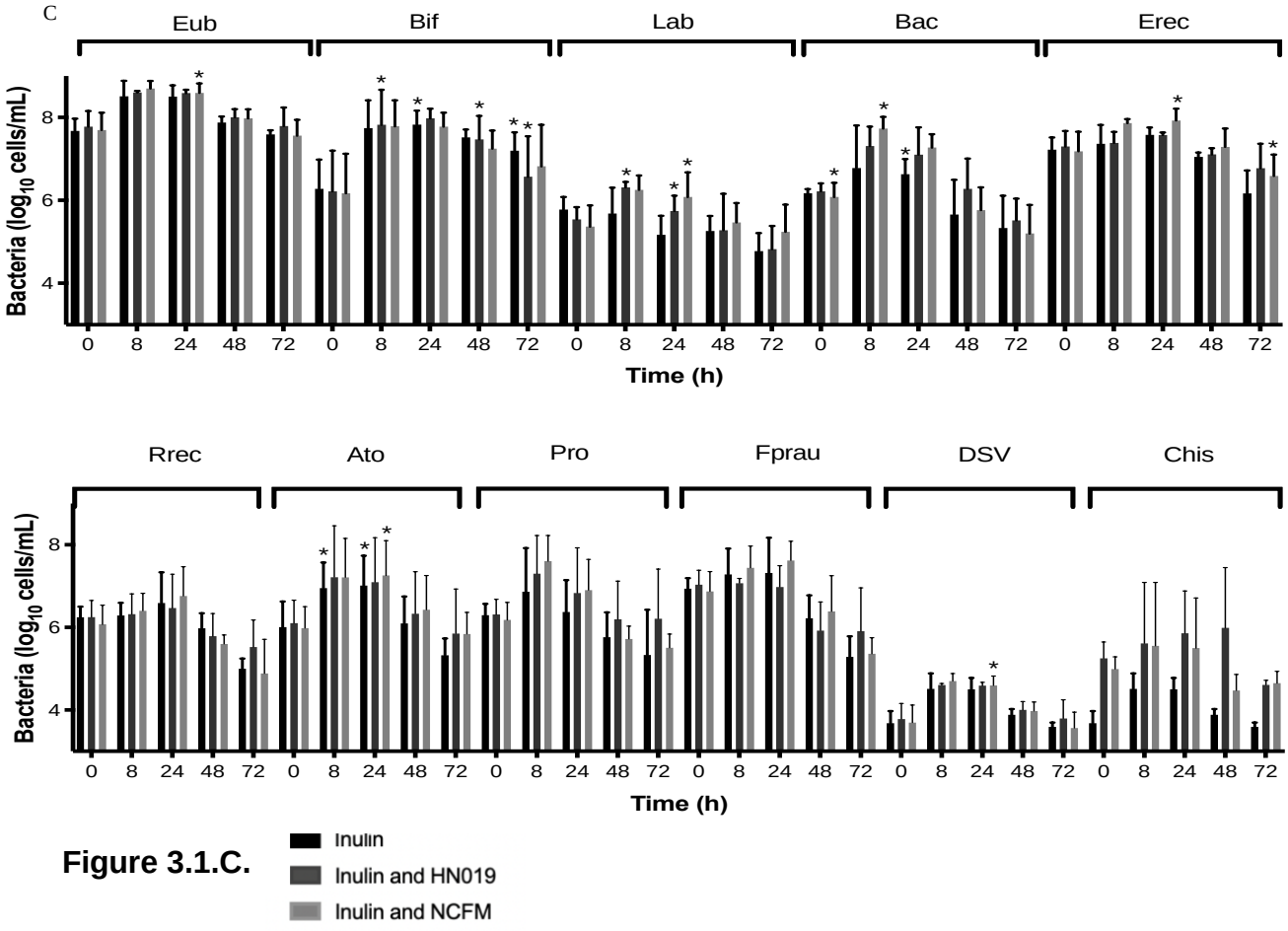

Figure 3.1.C.

Figure 3.1. Bacterial enumeration (log_{10} cells/mL) from *in vitro* batch culture after fermenting for 0, 8, 24, 48 and 72h. The bacteria analysed were total bacteria (Eub), *Bifidobacterium* spp. (Bif), lactic acid bacteria (Lab), *Bacteroidaceae* and *Prevotellaceae* (Bac), Clostridial cluster XIVa and XIVb (Erec), *Roseburia* (Rrec), *Atopobium* spp. (Ato), Clostridial cluster (Pro), *Faecalibacterium prausnitzii* (Fprau), *Desulfovibrionales* and *Desulfuromonales* (DSV) and *Clostridium histolyticum* group (Chis). Figure 3.1. A. highlights the results after fermenting WD (wheat dextrin), WD and HN019 (*Bifidobacterium lactis* HN019) and WD and NCFM (*Lactobacillus acidophilus* NCFM). Figure 3.1. B. shows bacterial counts after fermenting PHGG (Partially hydrolysed guar gum), PHGG and HN019 (*Bifidobacterium lactis* HN019) and PHGG and NCFM (*Lactobacillus acidophilus* NCFM). Figure 3.1. C. displays results after fermenting inulin, inulin and HN019 (*Bifidobacterium lactis* HN019) and inulin and NCFM (*Lactobacillus acidophilus* NCFM). Data are presented as mean ± SD (n=3) and *shows significant difference ($P \leq 0.05$) compared to the baseline (T0).

3.4.4 Effect of wheat dextrin in combination with *B. lactis* HN019 and *L. acidophilus* NCFM on organic acid production

Acetate production significantly increased at all timepoints in the WD ($P \leq 0.028$) and HN019 and WD ($P \leq 0.045$) vessels and NCFM and WD vessels ($P \leq 0.037$) (Figure 3.2A). Butyrate production significantly increased in the WD vessels ($P \leq 0.026$) and NCFM and WD vessels ($P \leq 0.046$) (Figure 3.2B). The WD vessels had the highest amount of acetate (increased by 102 mM from baseline) and butyrate (increased by 14.66 mM from baseline) compared to WD with probiotic strains. Propionate production was significantly increased in the WD, HN019 and WD and NCFM and WD vessels with a similar increase from baseline in each 41.6 mM, 54.99 mM and 45.81mM, respectively (Figure 3.2C).

3.4.5 Effect of partially hydrolysed guar gum in combination with *B. lactis* HN019 and *L. acidophilus* NCFM on organic acid production

Acetate was significantly increased in the PHGG vessels in all conditions with PHGG and HN019 significantly increasing at 24h (increased by 94.75mM from baseline, $P = 0.021$) until the end of incubation whereas, PHGG and PHGG with NCFM vessels increased significantly (increased by 94.75mM and 92.11mM from baseline, respectively)($P \leq 0.039$) and at 48h until the end of the study (Figure 3.2A). Butyrate production significantly increased from 24h ($P \leq 0.027$) until the end of the study at 72h ($P \leq 0.027$) in all PHGG conditions with very similar production across all PHGG vessels ranging from an increase of 20.43mM to 27.05mM (Figure 3.2B). Propionate production significantly increased at 24h and 72h in the PHGG vessels (increased by 37.29mM from baseline, $P \leq 0.046$), 24h, 48h and 72h in the PHGG and NCFM vessels (increased by 48.15mM from baseline, $P \leq 0.048$) and at 72h in the PHGG and HN019 vessels (increased by 44.8mM from baseline, $P = 0.036$) (Figure 3.2C).

3.4.6 Effect of inulin in combination with *B. lactis* HN019 and *L. acidophilus* NCFM on organic acid production

Inulin containing vessels significantly increased acetate at 8h (acetate increased by 49.08mM from baseline, $P = 0.021$) until the end of the incubation (acetate increased by 78.87mM from baseline, $P = 0.031$) whereas, the inulin and probiotic vessels did not generate significance until 48h ($P \leq 0.033$) which persisted until the end of the experiment ($P \leq 0.024$) (Figure 3.2A). Inulin and HN019 produced the highest amount of acetate with an increase of 107.36mM compared to baseline. Butyrate production was significantly elevated at 24h (increased by 26.94mM compared to baseline, $P = 0.036$) and remained elevated for the duration of the experiment (timepoint 48h had the highest increase in acetate by 33.3mM from baseline, $P = 0.010$) in the inulin vessels (Figure 3.2B). The only significant increase ($P = 0.049$) in propionate production was with inulin and NCFM vessels at 24h with a small increase from baseline of 14.18mM (Figure 3.2C).

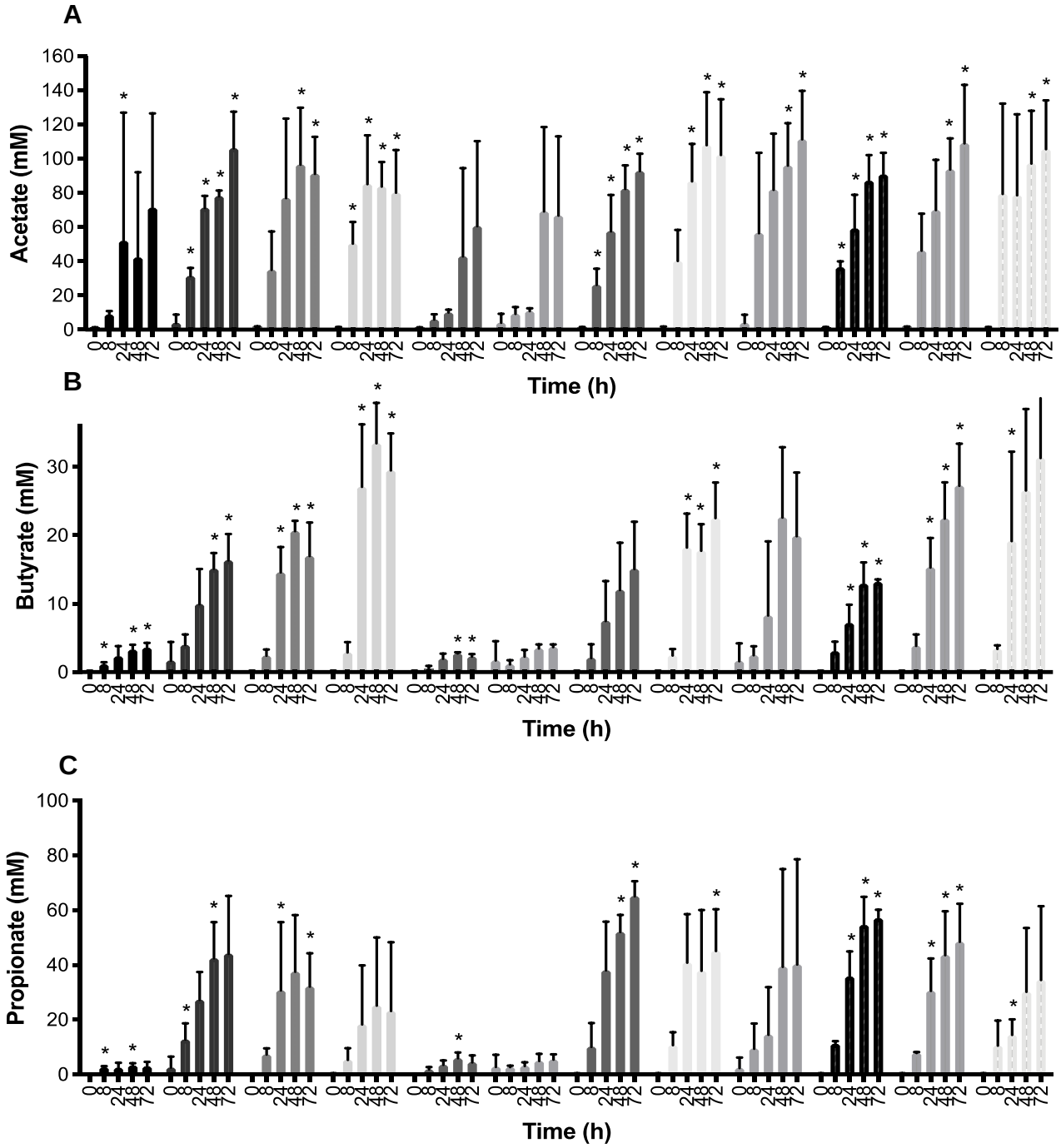

Figure 3.2. Organic acid production (mM) from *in vitro* batch culture fermentation of (from left to right) blank, WD (wheat dextrin), PHGG (Partially hydrolysed guar gum), inulin, HN019 (*Bifidobacterium lactis* HN019), NCFM (*Lactobacillus acidophilus* NCFM), WD and HN019, PHGG and HN019, inulin and HN019, WD and NCFM, PHGG and NCFM and inulin and NCFM at 0, 8, 24, 48 and 72 hours. Organic acids measured were acetate (A), butyrate (B) and propionate (C). Data are presented as mean ± SD (n=3) and * shows significant difference ($P \leq 0.05$) compared to the baseline (T0).

3.5 DISCUSSION

Overall, the main findings were that inulin when combined with a probiotic significantly prolonged the increase in bifidobacteria compared to inulin alone and significantly increased lactic acid bacteria, *Bacteroides* and *Clostridium coccoides–Eubacterium rectale.* Both WD and PHGG did not have any further benefit in terms of bacterial metabolism profile when combining the dietary fibre with a probiotic.

Inulin is a recognised prebiotic as it reaches the colon intact where it is then selectively fermented principally by bifidobacteria (Roberfroid et al., 1998). However, the degree of degradation depends on the specific strain of bifidobacteria and some strains are able to degrade all chain lengths (DP 12-25). The strain used in the present study is HN019 which has previously been grouped with *Bifidobacterium adolescentis* LMG 10734, which can degrade oligofructose with a low DP but not inulin (Moens *et al.*, 2016).

A recent study showed that HN019 degraded inulin at a slower rate in comparison to fructooligosaccharides (partially hydrolysed inulin (DP 2-8)) but was unable to degrade oligosaccharides greater than DP 7, therefore some of the inulin in the current study may have been degraded as the DP was from 2-65 (Sims *et al.*, 2014). Similarly, a study on 60 healthy volunteers with predisposition to constipation fed *B. lactis* GCL2505 and inulin and there was a significant increase in *B. longum* and *B. adolescentis*, but interestingly not *B. lactis* (Anzawa *et al.*, 2019). This was supported by Rossi *et al.* (2000) showing that the bifidobacterial strains *B. longum*, *B. thermophilus* and *B. adolescentis* were able to degrade inulin. This demonstrates that other bifidobacteria strains may need to be present in the colon to aid degradation of inulin and allow the HN019 strain to ferment the short chains once released (De Vuyst and Leroy, 2011; Falony *et al.*, 2009; Roberfroid *et al.*, 1998).

This aligns with the current findings as in the inulin vessels *Bifidobacterium* spp., was significantly elevated early in the study and higher than the PHGG and WD vessels,

resulting in early production of acetate via fructose-6-phosphate shunt and therefore significantly higher production of butyrate compared to baseline (Rossi *et al.,* 2005). It could also have been due to butyrate producing bacteria such as *Faecalibacterium prausnitzii, Eubacterium rectale–Clostridium coccoides* XIVa and XIVb and *Roseburia*. In this study, production may have been from *Eubacterium rectale–Clostridium coccoides* XIVa and XIVb as these were elevated during the study (Duncan *et al.,* 2002; Riviere *et al.,* 2016).

In the presence of inulin and HN019 a delayed increase in *Bifidobacterium* spp., and acetate production occurred compared to the inulin vessels therefore leading to an increase in butyrate production, but this was found to be insignificant. Therefore, the inulin may determine which bacterial metabolism occurs and a mixture of probiotics are important to increase influence on bacterial communities present.

Neither WD nor PHGG showed any further bacterial metabolism with the addition of HN019 or NCFM and there appeared to be no cross feeding to enhance bifidobacteria or lactic acid bacteria. However, as mentioned in a previous prebiotic fermentation experiment (Pyle *et al.,* 2020, unpublished), WD and PHGG both significantly increased in *Bacteroides* and *Clostridium* cluster IX, which have been shown to produce propionate, which was elevated in all vessels containing WD and PHGG but not inulin.

Propionate can play a role in appetite regulation by stimulating peptide tyrosine tyrosine and glucagon-like peptide-1 hormones which lead to a reduction in energy intake and therefore aid weight loss (Psichas *et al.*, 2015). This means that WD and PHGG may be effective for weight management. In 60 over-weight volunteers, propionate was delivered by an inulin-propionate ester resulting in weight reduction highlighting its ability for weight management (Chambers *et al.,* 2015). There have been synbiotic randomised, double-blind, placebo-controlled trials, RCT (Anzawa *et al.,* 2019; Childs *et al.,* 2014; Krumbeck *et al.,* 2018; Min *et al.,* 2012). To our knowledge no other study has combined *L. acidophilus* NCFM

and inulin or *B. lactis* HN019 and inulin. However, other RCT have used the above prebiotic and probiotics in different combinations and these have positively impacted common gastrointestinal problems.

The NCFM and HN019 strains combined with polydextrose have been found to shorten transit time in patients suffering from constipation and increase stool frequency in elderly volunteers after consuming NCFM and lactitol (Magro *et al.*, 2014; Ouwehand *et al.*, 2019). This was supported by Waitzberg *et al.* (2013) as improved transit time, stool consistency and shape was found after consuming fructooligosaccharides (FOS) with a mixture of probiotics: *L. paracasei* (Lpc-37), *L. rhamnosus* (HN001), *L. acidophilus* (NCFM) and *B. lactis* (HN019). Additionally, inulin in combination with the probiotics *S. thermophilus*, *L. bulgaricus* and *B. lactis* has been shown to decrease incidence of diarrhoea (Ringel-Kulka *et al.*, 2015). Furthermore, in adults, inulin in the form of a synbiotic can also reduce risk factors (high sensitivity C-reactive protein) for developing cardiovascular diseases and reduce serum insulin concentrations (Asemi *et al.*, 2014).

In children who were receiving antibiotic therapy, the risk of bacterial illness was reduced by 94.3% and an increased energy and weight gain occurred after consuming FOS and *L. acidophilus* (Schrezenmeir *et al.*, 2004). Similar findings were reported in 316 children consuming FOS, *L. acidophilus* and *Bifidobacterium* spp., with a reduction in constipation, days ill and increase in weight gain (Fisberg *et al.*, 2002).

Few studies above use a single strain as their probiotic intervention. More research is required to fully understand the relationship between each bacterial strain to assess the pathways and cross-feeding of bacteria within the gut to be able to optimise functionality.

3.6 CONCLUSION

Overall, the addition of probiotics HN019 and NCFM may help to enhance bacterial metabolism of inulin and cross feeding between bacteria allowing a prolonged increase in *Bifidobacterium* and lactic acid bacteria. However, WD and PHGG may be more optimal alone or in comparison with the strains HN019 and NCFM and may be important in weight management. Therefore, further analysis of inulin and HN019 and inulin and NCFM in a continuous three-stage gut model system will be used to assess the impact of the synbiotic at each stage of the colon.

3.7 ACKNOWLEDGEMENTS

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Appendix

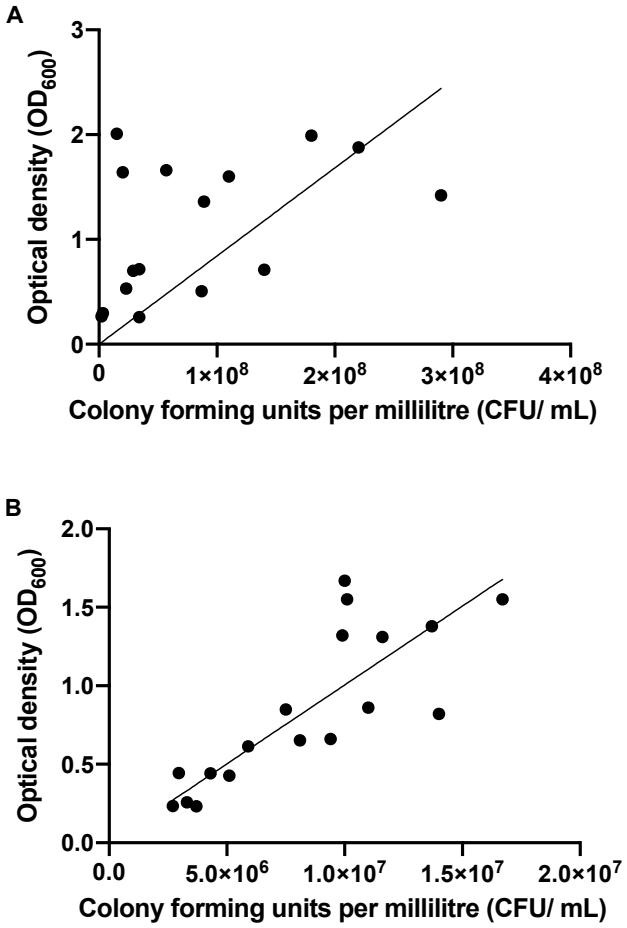

Figure 3.3. The growth curve of probiotic *B. lactis* HN019 (A) and *L. acidophilus* NCFM (B) correlating the optical density against the colony forming units per millilitre (CFU/mL). The figures show a range of concentrations of probiotic diluted with phosphate buffered saline grown on de Man-Rogosa-Sharpe for the NCFM strain and or de Man-Rogosa-Sharpe supplemented with 0.05% (w/v) cysteine for the HN019 strain after 24h in anaerobic conditions at 37ºC in triplicate.

4 Chapter 4: *Bifidobacterium lactis* HN019 or *Lactobacillus acidophilus* NCFM combined with inulin modifies bacterial metabolism but healthy and IBS-D donors differ in propionate and GABA productions during an *in vitro* three stage gut model system fermentation

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4.1 Abstract

Lactobacillus spp. and *Bifidobacterium* spp. have been shown to produce gamma-aminobutyric acid (GABA) which has an important role in the gut-brain axis and is the main inhibitory neurotransmitter. Irritable bowel syndrome (IBS) sufferers often have co-morbidity of anxiety and depression which may be improved with alterations in GABA. The aim of this study was to assess gut bacterial metabolism after anaerobic continuous culture fermentation of the prebiotic inulin alone or as a synbiotic combined with *Bifidobacterium lactis* HN019 (*B. lactis* HN019) and *Lactobacillus acidophilus* NCFM (*L. acidophilus* NCFM) and to predict the effect in different regions of the colon comparing healthy and IBS donors. A secondary aim was to investigate the presence of neurotransmitter GABA after prebiotic and synbiotic intervention. An *in vitro* gut model fermentation was run to simulate different colonic regions. The conditions were steady state 1 (SS1) no treatment, steady state 2 (SS2) inulin and steady state 3 (SS3) inulin and *B. lactis* HN019 or inulin and *L. acidophilus* NCFM. Samples were collected after SS1, SS2 and SS3 for bacterial enumeration, organic acid and GABA production through fluorescence *in-situ* hybridisation combined with flow cytometry, gas chromatography and liquid chromatography-mass spectrometry, respectively. *Bifidobacterium* spp., increased after inulin addition in all vessels with the healthy group having the greatest increase. Propionate production was significantly higher in the IBS-D group. The GABA concentration significantly

increased after the inulin intervention and synbiotic intervention in the healthy group. In conclusion, prebiotics have a more profound effect on the gut microbiota in comparison to probiotics. The GABA production after the synbiotic intervention may be an approach to elevate GABA production in healthy volunteers but more research is required on underpinning mechanisms in IBS sufferers to explain unaltered GABA production.

4.2 Introduction

Functional gastrointestinal (GI) disorders effect an individual's quality of life and are becoming increasingly common with one in four individuals in United Kingdom, United States and Canada meeting functional GI criteria (Palsson et al., 2020). One of the most common functional GI disorders is Irritable Bowel Syndrome (IBS) which 'is a chronic, relapsing and often lifelong disorder. It is characterised by abdominal pain or discomfort, which may be associated with defaecation and/or accompanied by a change in bowel habit' (NICE, 2008). Symptoms of IBS include stomach cramps, bloating, diarrhoea, constipation, increased gut sensitivity and problems with digestion (Dupont, 2014, Ringel et al., 2011). There are three types of IBS: IBS diarrhoea (IBS-D), IBS constipation (IBS-C) and IBS alternating (IBS-A)(sometime referred to as IBS mixed). The current classification for IBS is Rome IV which takes into consideration involvement of the gut-brain axis (Hellstrom and Benno, 2019). The gut-brain axis, more recently termed Microbiota-gut-brain axis, involves the bidirectional communication between the GI tract, the brain and the importance of the gut microbiota to maintain intestinal homeostasis (Baj et al., 2019). For example, there may be sensory visceral signals from the gut to the brain regulating mood and from the brain to the gut regulating gut function.

Even though the cause of IBS is still unknown, there is an association with anxiety and depression, in a meta-analysis IBS-D suffered the greatest compared to the other IBS subtypes (IBS-A and IBS-C) (Clarke et al., 2009, Lee et al., 2017).

The enteric nervous system (ENS) is found along the lining of the GI tract, it comprises of neurotransmitters and signalling pathways, regulating the GI function independently of the central nervous system (CNS) (Rao and Gershon, 2016). Serotonin (5-HT) is a neurotransmitter which has a major role in gastrointestinal function, 95% of human serotonin is found in the gut, where it can regulate smooth muscle function, intestinal secretion and pain perception (McLean et al., 2007, Moloney et al., 2014). Previous research has found that IBS-D sufferers had increased platelet-depletion in plasma concentrations of 5-HT in comparison with healthy controls; therefore decreased 5-HT reuptake is a characteristic of IBS-D sufferers (Atkinson et al., 2006). Chey et al. (2004) demonstrated a 5-HT antagonist drug helped to relieve visceral pain, increase transit time and reduce diarrhoea.

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS) and $GABA_A$ and $GABA_B$ receptors control the release of serotonin from the enterochromaffin cells. It is known that *Lactobacillus* and *Bifidobacterium* spp., can produce GABA (McLean et al., 2007, Yunes et al., 2016, Racké et al., 1995). In a study by Aggarwal et al. (2018) found a significant reduction in GABA in serum samples from IBS-D sufferers. The GABAergic system releases GABA and comprises of glutamic acid decarboxylase (GAD)(two types of GAD are GAD1 and GAD2), GABA-transaminase (GABA-T), GABA-receptor (GABA-R)(two types of receptors: $GABA_A$ and $GABA_B$) and GABA transporter (GAT) (Li et al., 2012). Aggarwal et al. (2018) found downregulation of GAD2 (synthesises GABA), $GABA_B$ and GAT-2 (GAT-2 is a type of GAT required for GABA uptake) in IBS-D sufferers compared to the healthy control group. In addition, *in vitro* HT-29 human colon adenocarcinoma cell line analysis found a significant increase in

proinflammatory cytokine markers (interleukin-1β, tumor necrosis factor-α, and interleukin-6).

Lactobacilli and bifidobacteria have been found to be lower in abundance IBS patients compared to healthy control volunteers (Kassinen et al., 2007, Malinen et al., 2005). A mouse study found that *Lactobacillus rhamnosus* JB-1 changed the $GABA_A$ and $GABA_B$ receptors in the brain and lead to a reduction in behaviours associated with anxiety and depression (Bravo et al., 2011). A study conducted on 340 IBS sufferers found that *Lactobacillus acidophilus* NCFM reduced abdominal pain and improved IBS Symptom Severity Score after 12 weeks of the intervention with a dose of either 1×10^9 or 1×10^{10} colony forming units (CFU) (Lyra et al., 2016). Additionally, anxiety and depression symptoms improved after the probiotic intervention but were not found to be significant. This suggests that a dietary intervention may improve the ratio of such beneficial bacteria and IBS symptoms may be improved with alterations in GABA production (Chassard et al., 2012, Codling et al., 2010, Kassinen et al., 2007, Barrett et al., 2012).

Therefore, using a prebiotic which is bifidogenic and increases the production of SCFA as well as the probiotic *Lactobacillus acidophilus* or *Bifidobacterium lactis* may be a possible treatment method for IBS sufferers. The addition of a prebiotic and probiotic is called a synbiotic, which can be used to enhance benefits from the prebiotic/probiotics alone.

To date, there is a limited amount of research on the use of synbiotics in IBS sufferers. Gracie and Ford (2015) conducted a review and found only four randomised control trials with three reflecting improvements in abdominal pain, bloating and increases in beneficial bacteria (Min et al., 2012, Rogha et al., 2014, Tsuchiya et al., 2004). More recently, a study in children found that synbiotic (*B. lactis* B94 5×10^9 CFU and inulin 0.9g twice a day) improved recovery from IBS symptoms (belching abdominal fullness, bloating after meals, constipation and mucus in the faeces) compared to prebiotic group alone (Basturk et al., 2016).

The aim of this study was to assess gut bacterial metabolism after anaerobic continuous culture fermentation of the prebiotic inulin alone or as a synbiotic combined with *Bifidobacterium lactis* HN019 and *Lactobacillus acidophilus* NCFM. A further aim was to investigate the concentration of the neurotransmitter gamma-aminobutyric acid after the prebiotic and synbiotic intervention.

4.3 Method

4.3.1 Subjects

Three healthy volunteers and three IBS-D donors (32±6 years old) donated faecal samples. The healthy volunteers had no history of gastrointestinal disorders and IBS-D volunteers had been diagnosed by a GP and completed a suitability questionnaire (Appendix. 1). None of the participants had consumed antibiotics in the last 3 months or prebiotic/probiotic in the previous 2 weeks. Ethical approval was obtained from University of Reading research ethics committee.

4.3.2 Three-stage gut model continuous culture colonic system

The colonic model system (GM) consists of three glass vessels which represents the three sections of the colon proximal (V1), transverse (V2) and distal (V3). Physicochemical conditions (gradients of pH, substrate concentration, flow rate) are replicated in this *in vitro* model which was validated in a study by Macfarlane et al. (1998). Two gut models were run in parallel with the same faecal sample to compare different synbiotic combinations. The parallel models were replicated in three different healthy donors and three different IBS-D donors with a total of 12 gut models ran in total.

4.3.3 Media

Autoclaved complex gut model medium (starch 25g, peptone water 25g, tryptone 25g, yeast extract 22.5g, NaCl 22.5g, KCl 22.5g, mucin 20g, casein 15g, pectin 10g, xylan 10g, arabinogalactan 10g, $NaHCO_3$ 7.5g, $MgSO_4$ 6.25g, guar gum 5g, inulin 5g, cysteine.HCl 4g, KH_2PO_4 2.4g, K_2HPO_4 2.4g, bile salts 2g, $CaCl_2.6H_2O$ 0.75g, $FeSO_4.7H_2O$ 0.025g, haemin 0.25g, Tween 80 5ml, vitamin K 50µl per 5 litres) (Sigma, St.Louis, MO) was added to each vessel (V1 51mL, V2 66mL and V3 82mL) and incubated overnight in anaerobic conditions (oxygen-free nitrogen at a rate of 15ml/min). Following this, the medium was then continuously pumped (V1 to V2 to V3) into each vessel at a rate of 6.25mL/h for the healthy donors and 12.5mL/h for the IBS-D donors (Camilleri et al., 2008). Once the medium had flowed from the 5l medium bottle to the waste container this equated to 1 turnover and 8 turnovers is usually when steady state is reached but this was confirmed by SCFA.

4.3.4 Faecal samples

Participants brought a fresh faecal sample (<3 hours) to the laboratory in anaerobic conditions (<1% O_2 and 9-13% CO_2) (AnaeroJarTM 2.5 L and AnaeroGemTM, Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK). The sample was diluted with phosphate buffer saline (PBS) 20% (w/v) (pH 7.4) and homogenised (Stomacher 400, Seward, West Sussex, UK) for 2 minutes at 240 paddle beats per minute. Vessels were inoculated with the faecal slurry: V1 28mL, V2 33mL and V3 37mL.

4.3.5 Steady state

The system then reached equilibrium (approximately eight turnovers) as measured through short chain fatty acid (SCFA) stabilisation which represented steady state one (SS1). After SS1, a second steady state (SS2) was carried out. This included feeding a prebiotic to the proximal

vessels every day and waiting for a further equilibrium period. The final stage involved adding a probiotic and prebiotic to the proximal vessel every day until steady state three (SS3) was reached.

4.3.6 Substrates

The SS1 was no treatment and contained only the faecal inoculum and GM medium. The SS2 was 4g of inulin being added to the proximal vessel in both parallel vessels. The SS3 was *Bifidobacterium lactis* HN019 ($3x10^8 \pm 3$ CFU/mL) and inulin (4g) added to the V1 (Condition A) and condition B was *Lactobacillus acidophilus* NCFM ($3x10^8 \pm 3$ CFU/mL) and inulin (4g) added to V1.

Inulin has an average DP of 12 (Roberfroid, 2005). The probiotic *Bifidobacterium lactis* HN019 and *Lactobacillus acidophilus* NCFM (Danisco Brazil, Cotia) were freeze dried and stored at -4^{o}C prior to use (GRAS, 2012, GRAS, 2010). The strains were grown in anaerobic conditions at 37^{o}C in de Man-Rogosa-Sharpe (MRS) (Oxoid Ltd, Basingstoke, Hampshire, UK) broth and de Man-Rogosa-Sharpe supplemented with 0.05% (w/v) cysteine (Sigma, St. Louis, MO), respectively. Bacteria were grown at different dilutions in triplicate for 24h to obtain $5x10^7 \pm 1x10^7$ CFU/mL (Appendix Figure 4.4.). The day prior to SS3 addition, strains were grown in the above conditions for 24h then on the day diluted with PBS and immediately added to the vessels. This was repeated each day for the 8 turnovers and counts were confirmed by growing the culture in MRS agar and MRS-C agar for 48-72h.

4.3.7 Samples

Samples were collected from each vessel on three consecutive turnovers prior to the sampling day to analyse SCFA stability (turnover 5, 6 and 7). Samples were immediately processed using an acidification method by Zhao et al. (2006) and run on turnover 7 using the gas

chromatography. If the SCFA were not stable, the experiment was continued until SCFA stabilisation. Once stable, samples were taken which was usually on turnover 8. The sample was used for bacterial enumeration through fluorescence *in situ* hybridisation flow-cytometry (FISH-FCM) (Table 4.1.) and organic acid production via gas chromatography followed Wang et al. (2019) methods. Additionally, samples were filtered (Corning, Costar, spin-x centrifuge tube filters, Sigma, St.Louis, MO) and detected for neurotransmitter GABA through liquid chromatography-mass spectrometry (LCMS-8050, Shimadzu, Buckinghamshire, UK) using a column (15cm x 2.1mm, 3 μm) of Discovery, HS, F5-3, (Supelco, Bellefonte, USA) and ran using the protocol of Shimadzu (2014).

Table 4.1. The bacterial probes names and DNA sequence used to detect common gut bacterial groups with validation references.

Probe name	Sequence (5' to 3')	Target group	References
Non Eub	ACTCCTACGGGAGGCAGC		(Wallner et al., 1993)
Eub338 I	GCT GCC TCC CGT AGG AGT	Most bacteria	(Daims et al., 1999)
Eub338 II	GCA GCC ACC CGT AGG TGT	Planctomycetales	(Daims et al., 1999)
Eub338 III	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	(Daims et al., 1999)
Bif164	CAT CCG GCA TTA CCA CCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*	(Langendijk et al., 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis;* all *Vagococcus, Pediococcus* and *Paralactobacillus* spp, *Melisococcus, Tetragenococcus, Catellicoccus, Enterococcus*	(Harmsen et al., 1999)
Bac303	CCA ATG TGG GGG ACC TT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz et al., 1996)
Erec482	GCT TCT TAG TCA RGT ACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb)	(Manz et al., 1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II)	(Franks et al., 1998)
Rrec584	TCA GAC TTG CCG YAC CGC	Roseburia subcluster	(Franks et al., 1998)
Prop853	ATT GCG TTA ACT CCG GCAC	*Clostridial* cluster IX	(Walker et al., 2005)
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium, Colinsella, Olsenella* and *Eggerthella* spp.; *Cryptobacterium curtum; Mycoplasma equigenitalium* and *Mycoplasma elephantis*	(Harmsen et al., 2000)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	(Hold et al., 2003)
DSV687	TAC GGA TTT CAC TCC T	Most Desulfovibrionales (excluding Lawsonia) and many Desulfuromonales	(Devereux et al., 1992)

4.3.8 Statistical analysis

All statistical results were analysed in SPSS version 25 (SPSS Inc, Chicago, Ill, USA). The FISH-FCM, organic acid and neurotransmitter production were analysed using two-way mixed ANOVA to compare different test substrates, subjects and time points. Where significant differences were found, a post hoc analysis was performed using Tukey multiple comparison tests. A paired t-test was used to further analyse the difference between vessels at each time point. Statistical analysis was accepted at $P < 0.05$ for all analyses.

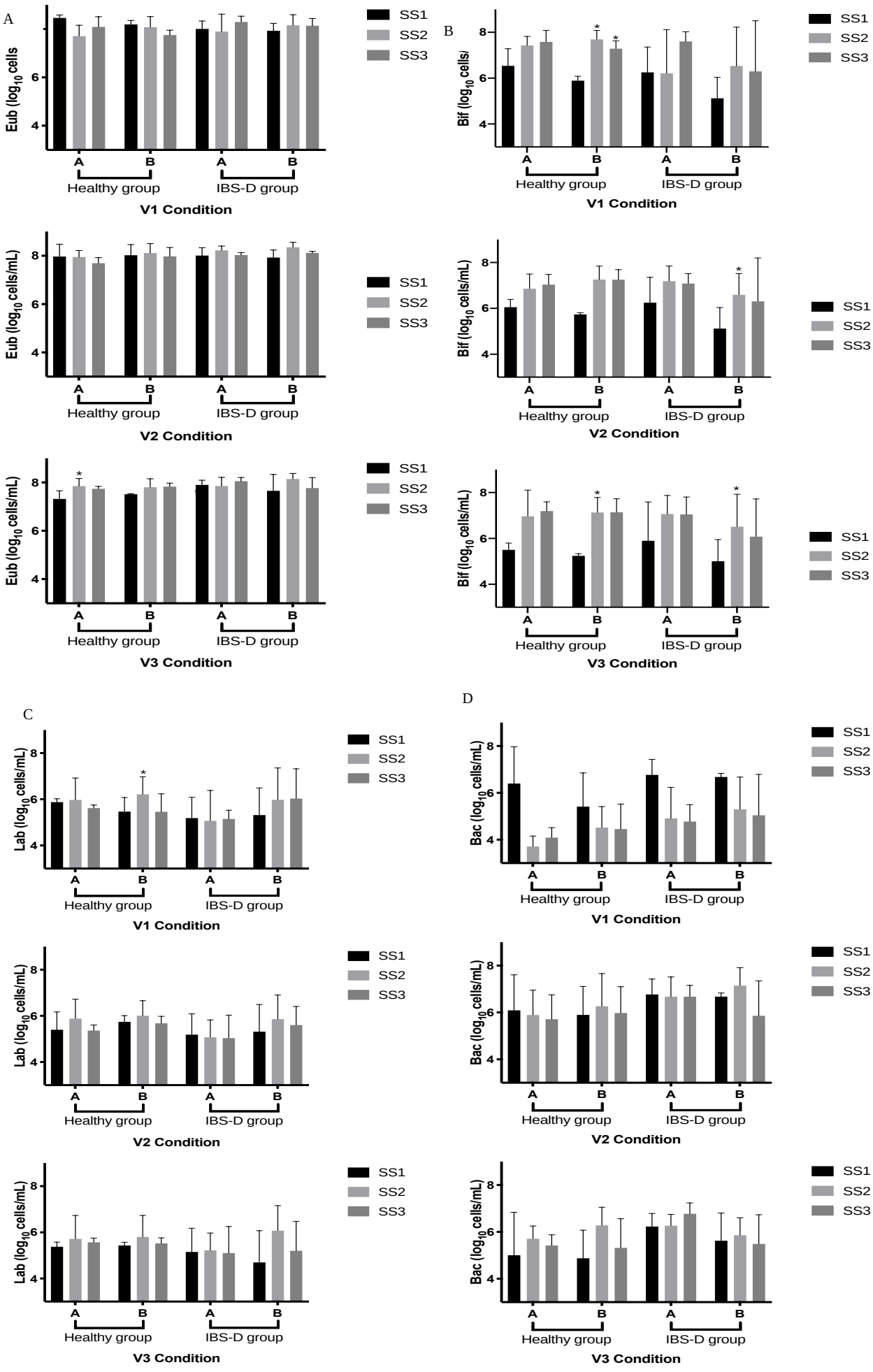
4.4 Results

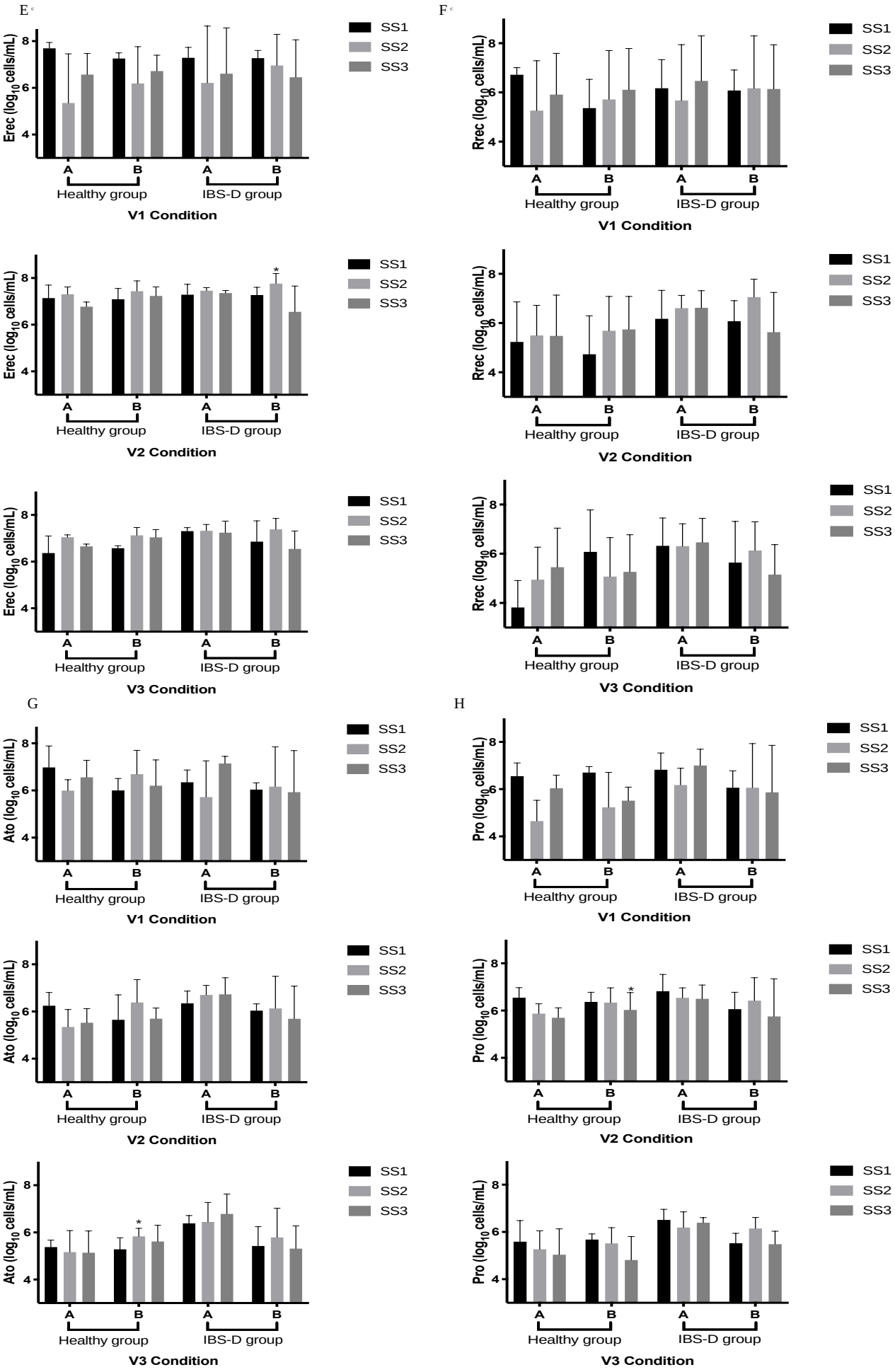
4.4.1 Bacterial enumeration

The healthy and IBS-D group initially had very similar bacterial profiles. However, the healthy group had a significant lower number of total bacteria in the distal vessels compared to the IBS-D group (difference of 0.59 $\log_{10}$ cells/mL $P = 0.022$) (Figure 4.1A). Generally, the distal vessels had significantly reduced bacteria compared to the proximal and transverse vessels, especially in the healthy group.

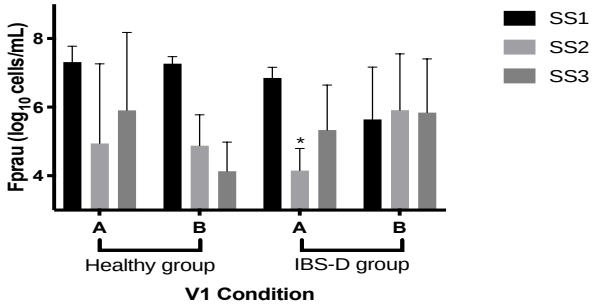
Both groups significantly increased (condition B $P \leq 0.046$) in *Bifidobacterium* spp., after the inulin intervention with all vessels within the healthy group having the greatest increase by 1.89 $\log_{10}$ cells/mL compared to 1.5 $\log_{10}$ cells/mL in the IBS-D group (Figure 4.1B). Generally, *Bifidobacterium* spp., did not significantly increase further following the probiotic intervention and had a significant decrease (proximal vessels found a 0.4 $\log_{10}$ cells/mL decrease $P = 0.010$) in the healthy group when *L. acidophilus* NCFM was added.

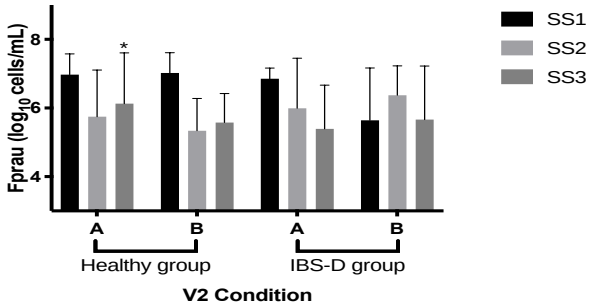
In the healthy and IBS-D groups initial enumeration of bacteroides ($P \leq 0.013$) and in the healthy group *Clostridium cluster* IX ($P \leq 0.038$) were significantly higher in the proximal vessels compared to the distal vessels (Figure 4.1D&H). Conversely, following the inulin intervention there was a shift showing a significantly higher bacteroides ($P \leq 0.006$) and *Clostridium cluster* IX ($P \leq 0.038$) count in the distal vessels compared to the proximal. Healthy volunteers had a marked increase in bacteroides in the distal vessels following the inulin intervention when compared to the proximal vessels, showing an average increase of 1.88 $\log_{10}$ cells/mL compared to the IBS-D group which increased by an average of 0.96 $\log_{10}$ cells/mL.

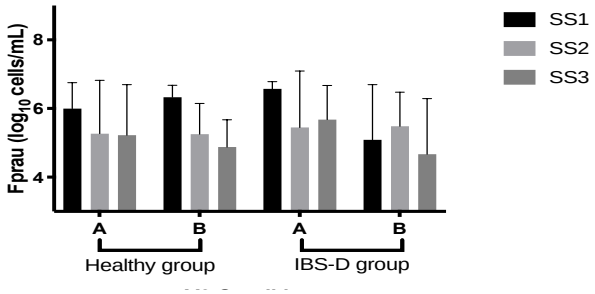

A
B
C
D
SS1
SS2
SS3
Eub (log10 cells
Bif (log10 cells/
Eub (log10 cells/mL)
Bif (log10 cells/mL)
Lab (log10 cells/mL)
Bac (log10 cells/mL)
Healthy group
IBS-D group
V1 Condition
V2 Condition
V3 Condition


E
F
G
H
SS1
SS2
SS3
Erec (log10 cells/mL)
Rrec (log10 cells/mL)
Ato (log10 cells/mL)
Pro (log10 cells/mL)
8
6
4
A
B
Healthy group
IBS-D group
V1 Condition
V2 Condition
V3 Condition
*

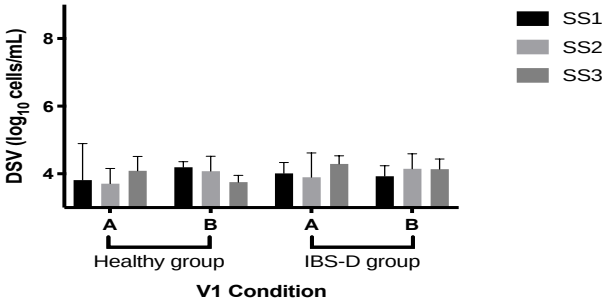
I

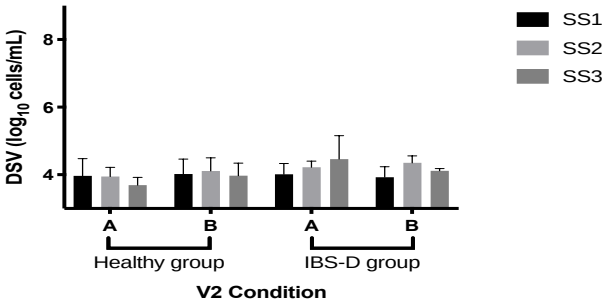

Fprau (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
V1 Condition
SS1
SS2
SS3

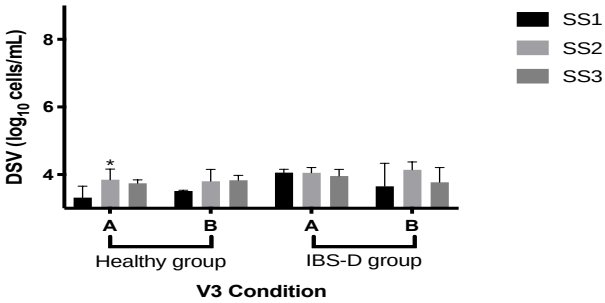

Fprau (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
V2 Condition
SS1
SS2
SS3


Fprau (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
SS1
SS2
SS3

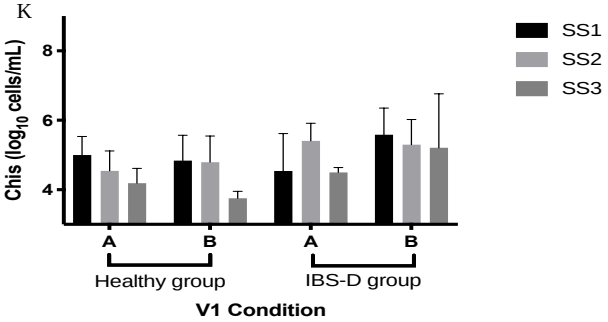
J

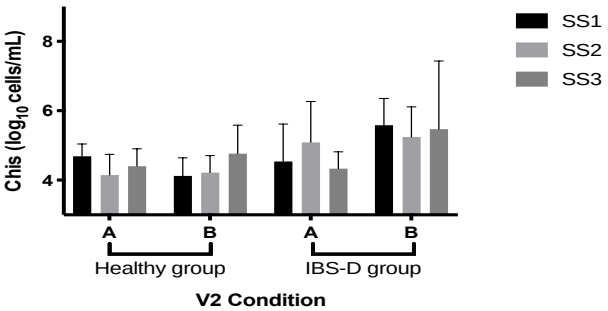

DSV (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
V1 Condition
SS1
SS2
SS3


DSV (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
V2 Condition
SS1
SS2
SS3


DSV (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
V3 Condition
SS1
SS2
SS3

K


Chis (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
V1 Condition
SS1
SS2
SS3


Chis (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
V2 Condition
SS1
SS2
SS3

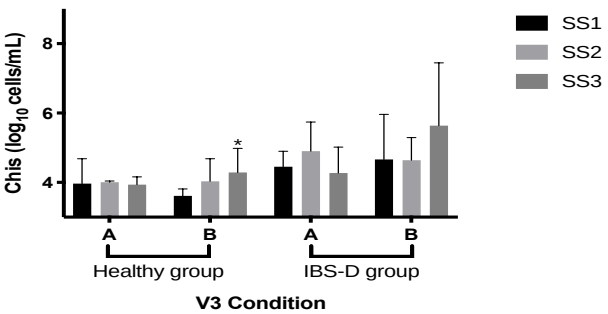

Chis (log10 cells/mL)
8
6
4
A
B
A
B
Healthy group
IBS-D group
V3 Condition
SS1
SS2
SS3

Figure 4.1. Bacterial enumeration (log_{10} cells/mL) from *in vitro* gut model systems after steady state one (SS1) no treatment, steady state two (SS2) prebiotic intervention and steady state three (SS3) synbiotic intervention. The gut model system comprises of three vessels: vessel one (V1) representing the proximal colon, vessel two (V2) simulating the transverse colon and vessel three (V3) representing the distal colon. A and B show different interventions whereby condition A is supplemented with inulin and ***B. lactis*** HN019 and condition B inulin and ***L. acidophilus*** NCFM. The healthy participants are represented on the left and the irritable bowel syndrome diarrhoea on the right. The bacteria analysed were total bacteria (Eub, A), *Bifidobacterium* spp. (Bif, B), lactic acid bacteria (Lab, C), *Bacteroidaceae* and *Prevotellaceae* (Bac, D), *Clostridial* cluster XIVa and XIVb (Erec, E), *Roseburia* (Rrec, F), *Atopobium* spp. (Ato, G), *Clostridial* cluster IX (Pro, H), *Faecalibacterium prausnitzii* (Fprau, I), Desulfovibrionales and Desulfuromonales (DSV, J) and *Clostridium histolyticum* group (Chis, K). Data are presented as mean ± SD (n=3) and * shows significant difference ($P \leq 0.05$) compared to the previous steady state. For example, comparing SS1 to SS2 and SS2 to SS3.

4.4.2 Organic acids

Following the inulin intervention there was a reduction in propionate production in the healthy group within the proximal vessel, reducing by an average of 57.72mM compared to an average reduction of 19.29mM in the IBS-D group (Figure 4.2C). In contrast, in the IBS-D group there was an increase in propionate production following the inulin intervention within the transverse vessel, with an average increase of 13.73mM compared to an average increase of 4.59mM in the healthy group.

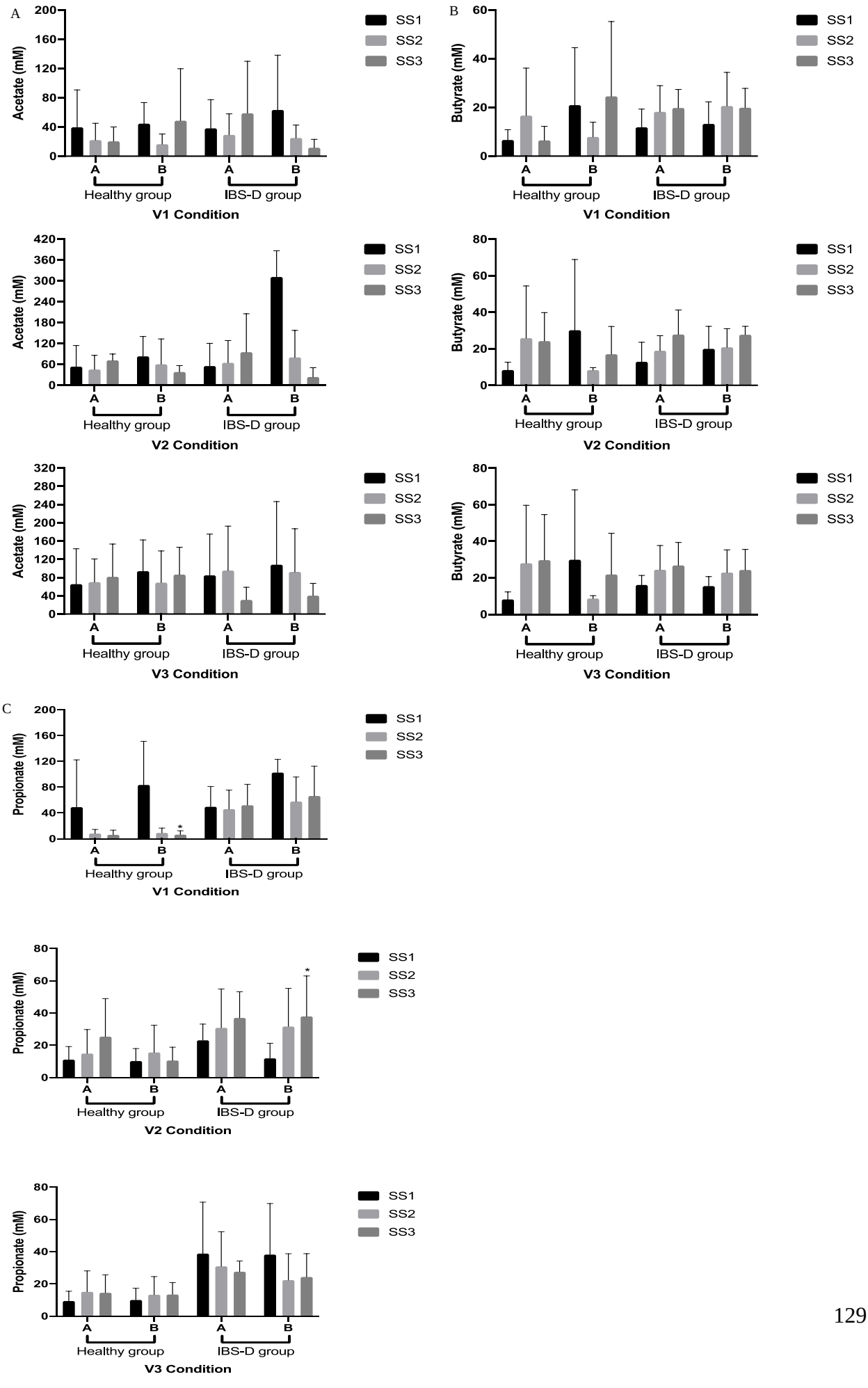

A
Acetate (mM)
Healthy group
IBS-D group
V1 Condition
V2 Condition
V3 Condition
SS1
SS2
SS3
B
Butyrate (mM)
C
Propionate (mM)

Figure 4.2. Organic acid production (mM) from *in vitro* three-stage gut model systems after steady state one (SS1) no treatment, steady state two (SS2) prebiotic intervention and steady state three (SS3) synbiotic intervention. The gut model system comprised of three vessels: vessel one (V1) representing the proximal colon, vessel two (V2) presenting the transverse colon and vessel three (V3) representing the distal colon. A and B show different interventions whereby condition A is supplemented with inulin and ***B. lactis*** HN019 and condition B inulin and *L. acidophilus* NCFM. The healthy participants are represented on the left and the irritable bowel syndrome diarrhoea on the right. Organic acids measured were acetate (A), butyrate (B) and propionate (C). Data are presented as mean ± SD (n=3) and * shows significant differences ($P \leq 0.05$) compared to the previous steady state. For example, comparing SS1 to SS2 and SS2 to SS3.

4.4.3 Neurotransmitters

In the proximal vessel the concentration of GABA was significantly higher ($P \leq 0.0244$) in the healthy group compared to the IBS-D group across both interventions with the healthy group having a 3996.42 ng/mL increase compared to the IBS-D group following the inulin and *L. acidophilus* NCFM intervention (Figure 4.3). In the healthy group GABA production increased following the inulin intervention, while both synbiotic interventions significantly increased ($P \leq 0.0319$) GABA in the proximal vessel when compared to SS1. The *B. lactis* HN019 and inulin intervention increased by 1720.05 ng/mL and the *L. acidophilus* NCFM and inulin increased by 3683.31 ng/mL when compared to SS1.

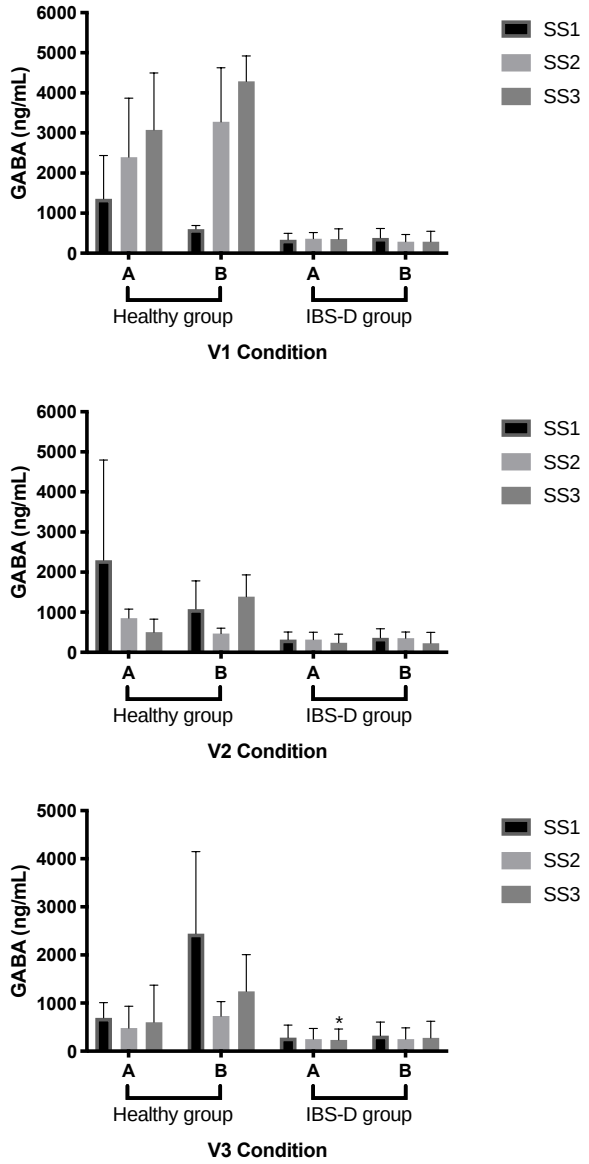


Figure 4.3. Neurotransmitter gamma aminobutyric acid (GABA, ng/mL) in an *in vitro* three-stage gut model system after steady state one (SS1) no treatment, steady state two (SS2) prebiotic intervention and steady state three (SS3) synbiotic intervention. The gut model system comprised three vessels: vessel one (V1) representing the proximal colon, vessel two (V2) presenting the transverse colon and vessel three (V3) representing the distal colon. A and B show different interventions whereby condition A was supplemented with inulin and ***B. lactis*** HN019 and condition B inulin and ***L. acidophilus*** NCFM. The healthy participants are represented on the left and the irritable bowel syndrome diarrhoea on the right. Data are presented as mean ± SD (n=3) and * shows significant difference ($P \leq 0.05$) compared to the previous steady state. For example, comparing SS1 to SS2 and SS2 to SS3.

4.5 Discussion

Initially, there was no significant difference in total bacteria between the IBS-D group and the healthy group. Previous research has shown that IBS-D volunteers have reduced amounts of *Bifidobacterium* and *Lactobacillus* (Malinen et al., 2005, Carroll et al., 2012). Generally, the distal vessels had significantly lower bacteria compared to the proximal and transverse vessels. This may be due to the distal colon having a lower nutrient availability (Macfarlane et al., 1998).

4.5.1 Bacterial metabolism following the inulin intervention

Following daily supplementation of inulin there was a significant increase in *Bifidobacterium* spp., in both the healthy and IBS-D group. This was expected as inulin is a well-known prebiotic (Gibson and Roberfroid, 1995). There is currently debate in the literature whether inulin would be a suitable intervention for IBS-D sufferers as there is a suggestion that low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) should be used (Nanayakkara et al., 2016, Huaman et al., 2018). The research states that prebiotics lead to enhanced gas production (Murray et al., 2014). However, the study showing higher gas production gave volunteers 40 g of prebiotic which exceeds the recommended daily amount of 30g per day in the United Kingdom (Hooper et al., 2015). The majority of human studies using inulin intervention consume 10-15g per day, which have been shown to be well-tolerated and shown to be selective for *Bifidobacterium* spp. (Bonnema et al., 2010). *Bifidobacterium* spp. are non-gas producing bacteria therefore if taken at adequate amount no further increase in gas production should occur (Turroni et al., 2009). In the current study, gas production in the IBS-D group was not significantly higher than the healthy group (Appendix 4.3). Similarly, healthy and IBS sufferers had similar changes in response to inulin but did not develop symptoms suggesting abnormal visceral sensation rather than altered fermentation

may be responsible for IBS symptoms (Eswaran et al., 2017). Forty IBS sufferers with abdominal functional distension were assigned to either a low FODMAP diet or B-GOS prebiotic supplementation and it was found that both conditions reduced gas production which was maintained following B-GOS intervention (Huaman et al., 2018). Thus, prebiotic supplementation may be an effective long-term intervention for IBS sufferers.

Interestingly, after inulin addition, *Clostridium* cluster IX and *Bacteroides* counts were significantly higher in the distal vessels compared to the proximal and transverse vessels. This may have been due to out-competing of other genera along with the *Bacteroides* preferring a higher pH (pH 6.5) (Walker et al., 2005).

4.5.2 Bacterial metabolism after synbiotic intervention

Synbiotic intervention did not seem to impact gut bacteria compared to the prebiotic alone, but this may have been due to the FISH-FCM technique as analyses of bacterial populations were at group level. Previous synbiotic studies in IBS volunteers have combined multiple probiotic strains which may lead to alterations at the group level (Tsuchiya et al., 2004, Noorbakhsh et al., 2019, Lee et al., 2019).

4.5.3 Organic acid production

SCFA help to maintain health of the host (Gao et al., 2009, Blouin et al., 2011, Samuel et al., 2008). They are transported around the body in the blood to organs or utilised by colonocytes. They enter cells by three methods: passive diffusion, carrier mediated transportation (sodium-coupled monocarboxylate transporter 1 or monocarboxylate transporter) or by activating G-protein-coupled receptors (GPCRs) (Sun et al., 2017). These signals can regulate metabolism, inflammation and disease. The most abundant SCFA in the colon are acetate (C_2), propionate (C_3) and butyrate (C_4) which account for 95% of all SCFA in the gut (Sun et al., 2017). The majority of SCFA are found in the proximal simulation of the colon as conditions (pH and

concentration of nutrients) are more optimal for bacterial fermentation hence, a greater concentration of SCFA (Sun et al., 2019).

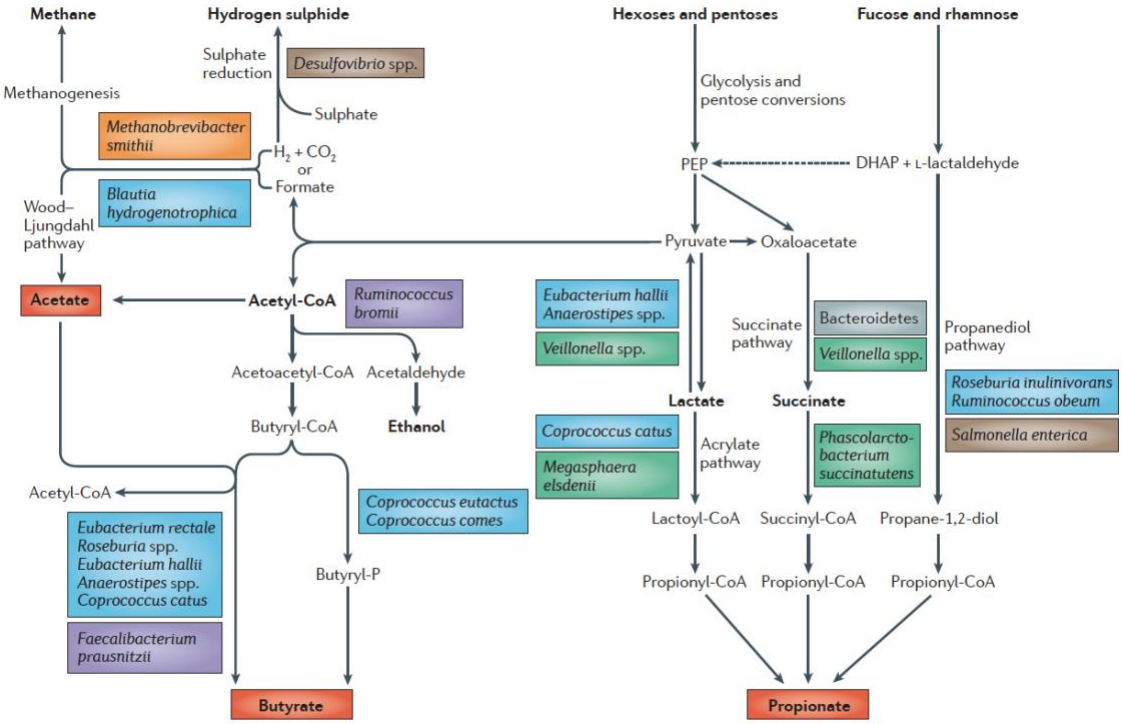


Figure 4.4. The pathways of microbial metabolites following carbohydrate fermentation and bacterial cross-feeding (Louis et al., 2014).

In the current study, there was a significantly higher concentration of propionate in the IBS-D group gut models at the start of the experiment compared to the healthy group gut models. Previous studies have found similar results showing a significantly higher level of propionate in 26 IBS sufferers compared to 26 control volunteers (Tana et al., 2010). Farup et al. (2016) found a trend towards a higher concentration of propionate in the IBS group compared to control. Also, IBS-D had a higher concentration of propionate compared to IBS-A and IBS-C (Valeur et al., 2016).

Propionate is predominantly produced through the succinate pathway by *Bacteroides* and some *Negativicutes* (Also known as *Veillonellaceae* or *Clostridium* cluster IX) but can also

occur via the lactate pathway by Firmicutes (*Negativicutes* and *Lachnospiraceae*) (Reichardt et al., 2014, Macy, 1979).

In addition, participants with higher propionate levels in the Tana et al. (2010) study were associated with more severe gastrointestinal symptoms, lower quality of life and negative emotions compared to those who had lower propionate levels. The propionate producing bacteria proteobacteria have been linked to an increased mental component, higher pain threshold and a higher proportion of proteobacteria found in IBS sufferers compared to healthy controls (Jeffery et al., 2012, Saulnier et al., 2011). In our study, *Clostridium* cluster IX did remain elevated in the IBS group compared to healthy group in the proximal and distal colon simulations but this was not significantly significant.

The majority of IBS sufferers have a co-morbidity of anxiety and depression with a prevalence of depression around 84% and anxiety 44% (Banerjee et al., 2017, Bravo et al., 2011). A meta-analysis involving 27 studies by Lee et al. (2017) found that participants suffering from IBS, had significantly higher levels of depression and anxiety compared to healthy participants and interestingly, IBS-D suffered the greatest with anxiety and depression compared to the other IBS subtypes (IBS-A and IBS-C).

4.5.4 Neurotransmitters

The gut brain axis is the bidirectional communication between the gut and the brain (Rhee et al., 2009). Communication occurs through the immune system, neuroendocrine system, autonomic and enteric nervous system (Rieder et al., 2017). Previous *in vitro* research has shown that probiotic lactobacilli and bifidobacteria can produce GABA (Barrett et al., 2012). GABA is the main inhibitory neurotransmitter of the central nervous system (CNS) and has been linked to anxiety and depression (Rieder et al., 2017). Production of GABA occurs through the GABA shunt which requires glutamine to be converted to glutamate which is a precursor of GABA (Connors et al., 2018, Baj et al., 2019). Glutamine is an essential amino

acid and required as an energy source for intestinal cells (Calder and Yaqoob, 1999). If depleted, it can lead to increased intestinal cell atrophy and permeability as it may directly be required for claudin-1 expression which is a tight junction protein (Bertrand et al., 2016). Glutamine has been shown in a randomised control trial to increase tight junction proteins after 8 week supplementation in 54 IBS-D sufferers and to reduce stool frequency (Zhou et al., 2019).

In the current study, GABA significantly decreased in the IBS-D and did not alter after prebiotic or synbiotic addition whereas, in the healthy group inulin intervention significantly increased GABA and the synbiotic increased it further. GABA production may not have increased in the IBS-D group as there may have been a limited amount of glutamate. L-glutamate may have been broken down through the 3-methylasparate pathway instead of the 4-aminobutyrate pathway which utilises glutamate without producing GABA (Louis and Flint, 2017). Pathways in which GABA may play a role in the immune response and bacterial metabolites are not well known. There may be an association between increased propionate and an inhibition of GABA. However, further research is required to assess the underpinning mechanisms.

4.6 Conclusion

Prebiotics may have a larger impact than probiotics on the gut bacteria by significantly altering the function of the gut at a genus level. Further probiotic addition may have impacted the gut microbiota at a species level. Combining multiple strains may give a further functional impact on bacterial metabolism. Synbiotic intervention may be an approach to elevate GABA production in healthy volunteers but more research is required to detect the underpinning mechanisms within IBS sufferers before it can be utilised as a dietary intervention.

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Appendix 4.1.

Participant information sheet

Stool sample donation from Irritable Bowel Syndrome (IBS) sufferers for *in vitro* systems modelling the human colon

No intervention is to be carried out. You are simply being asked to donate a stool sample to aid research. Before you decide if you want to take part it is important that you understand what is involved. Please read the following information and discuss with others if you wish. Please ask us if there is anything you do not understand or if you would like any additional information. Take time to decide whether or not you wish to take part. Your participation is entirely voluntary. This project has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee, and has been given a favourable ethical opinion for conduct.

Aim

This is an *in vitro* study which means that we will use your stool sample to assess the effects of different foods on the bacteria from your stool sample within laboratory experiments.

Volunteers are required to donate a bounty bar sized stool sample (~ 30g). Before you decide whether to donate this sample, please read the following information carefully. If you want to know anything about the study, which is not written here, please ask the investigator.

Why is this study being carried out?

- Bacteria play an important role in maintaining gut health. Therefore, by changing the gut bacteria community we may be able to impact on health. This current study will look at the impact of different foods on the gut microbial community to assess if any foods may potentially be of benefit to IBS sufferers.

Inclusion criteria/exclusion criteria

Inclusion criteria- If the following applies to you, you will be considered for participation in the trial:

- Aged 18 – 80 years old
- Diagnosed by GP as having IBS
- In good general health
- Provided written informed consent and be willing to participate in this study.

Exclusion criteria - If the following applies to you, you will be unable to participate in the trial:

- Received antibiotics in the previous three months.
- Consumed probiotic or prebiotic preparations on a regular basis (at least 3 times per week) in the last 2 weeks. (Examples of these food products are: Danone Actimel yoghurt drink, Yakult milk drink, Danone Activia yoghurt, Kellogg's rice crispies multigrain, Weetabix weetaflakes, Muller Vitality Yoghurt/Drinks, Warburton's healthy inside bread).
- Former participation in another study involving prebiotic or probiotic preparations or investigational drugs within the previous 1 month, or intention to use such drugs during the course of the study (please note sensory evaluations are still permitted).
- Currently prescribed immunosuppressive drugs. Participants will be required to withdraw should they begin taking any of the ineligible medication.
- *Intention* to use *regularly* other medication which affects gastrointestinal motility and/or perception.
- History of alcohol or drug misuse.
- Suffer from any major conditions involving the following:
- Head, Ears, Eyes, Nose and Throat
- Dermatological/Connective tissue
- Neurological
- Lymphatic
- Urogenital/Rectal
- Abdominal
- Respiratory
- Cardiovascular
- Incontinence

What will I be asked to do?

- All participants will be asked to fill out a health screening questionnaire and inclusion/exclusion criteria will be reviewed for volunteer eligibility
- If you are happy to participate you will be asked to sign a consent form
- Volunteers will be provided with pots in which to provide a faecal sample – we need this within two hours of voiding, thus after voiding volunteers will be required to deliver their sample to the Department of Food and Nutritional Sciences, University of Reading

Are there any risks?

This is an *in vitro* study. Volunteers are only required to provide a stool sample for a single study and no treatment will be provided, as such, participation in this study does not pose any significant risks.

Confidentiality
Confidentiality will be maintained by allocating volunteers an identification code, which will be used to identify all samples and data obtained. Volunteer's names will not be used in any reports or publications. All data generated from the study will be held securely within a password protected file, only the study investigators will have access to this a record of the names of the volunteers will not be held on the same file. Information matching volunteer names with identification codes will be kept by a departmental secretary in a locked filing cabinet, the investigators will only use identification codes. The
only time data will be matched with volunteer names is for those volunteers that request to have their personal results discussed with them. A request for individual results to be discussed will include a review of all sample results for the individual volunteer. A list of the names and addresses of the subjects in this project will be compiled, this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

General Information

- The analysis of the stool sample will occur at the University of Reading.
- If at any time you wish to withdraw from the study you are completely free to do so without giving a reason.
- The information collected will be used for research purposes only. All information will be confidential and individuals'names will not be used in any reports resulting from this work.
- Once the study has been completed you can request your results.
- All unused stool samples will be destroyed after the completion of the study and sample analysis.

This study has been approved by the Research Ethics Committee at Reading University. If you have any concerns over how this study has been conducted; please don't hesitate to contact the Head of Food and Nutritional Sciences.
Prof. Richard Frazier
r.a.frazier@reading.ac.uk

The investigators thank you for taking time to read this. If you have any queries please feel free to contact:
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phone
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Appendix 4.2.

Health and lifestyle questionnaire

Project Title:
Stool sample donation from Irritable Bowel Syndrome (IBS) sufferers for *in vitro* systems modelling the human colon

Name: _____

DOB: _____

Gender: _____

Address: _____

_____

_____

_____

_____

Telephone: _____

Height: _____ (cm)

Weight: _____ (kg)

Waist circumference: _____ (cm)

Questions to assess general health

Does the following apply to you?

	Yes	No	Don't know	If Yes – Please describe
Excessive Alcohol consumption (>14 units per week)				
Regular smoker				
Pregnancy, lactation or planning pregnancy				
Involvement in drug/ medication study in last month				
Use of antibiotics in the previous 6 months				

Please state any prescribed medication you are currently taking below:

_____
_____
_____
_____
_____

Do you have any food intolerances?

_____
_____
_____

Questions to assess IBS status:

	Yes	No	Don't know	If Yes – Please describe:
Have you been diagnosed with IBS by a GP?				
Do you consume probiotics or prebiotics regularly?				

Please state whether you predominantly suffer from diarrhoea or constipation (or a mix of both)

_____

_____

Please describe how often you have IBS attacks – i.e. monthly, weekly etc.

_____
_____

Are you aware of anything that triggers your IBS (e.g. certain foods (please state), stress)

_____
_____
_____

Do you suffer from any conditions involving the following?

	Yes	No	Don't know	If Yes – Please describe
Cardiovascular				
Abdominal				
Urogenital/Rectal				
Gastroenterological				
Lymphatic				
Neurological				
Coeliac disease				

All details will be kept strictly confidential

Thank-you for completing this questionnaire

Appendix 4.3.

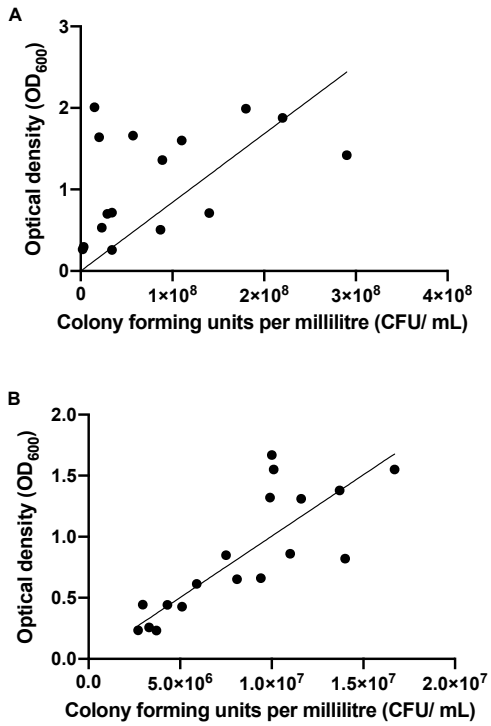

Figure 4.5. The growth curve of probiotic *B. lactis* HN019 (A) and *L. acidophilus* NCFM (B) correlating the optical density against the colony forming units per millilitre (CFU/mL). The figures show a range of concentrations of probiotic diluted with phosphate buffered saline grown on de Man-Rogosa-Sharpe for the *L. acidophilus* NCFM strain and de Man-Rogosa-Sharpe supplemented with 0.05% (w/v) cysteine for the *B. lactis* HN019 strain after 24h in anaerobic conditions at 37ºC in triplicate.

5 Chapter 5: Bacterial metabolism of zinc carnosine in an *in vitro* gut model fermentation

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5.1 Abstract

Zinc carnosine (ZnC) comprises of a zinc ion and L-carnosine. Studies have previously shown it to improve gut integrity and remission from Inflammatory Bowel Disease (IBD). However, no study to date has assessed the impact of ZnC on gut microbial metabolism. Therefore, the aim of this study was to assess whether ZnC combined with dietary fibre (wheat dextrin (WD) and partially hydrolysed guar gum (PHGG)) alter bacterial metabolism compared to dietary fibre alone and/or ZnC alone. *In vitro* gut model batch culture fermentations were run for 72 hours. Samples were collected for bacterial enumeration and organic acid production through fluorescent *in situ* hybridisation combined with flow cytometry and gas chromatography, respectively. Overall, the main findings were that the ZnC supplementation combined with WD increased in *Bifidobacterium* spp., and *Clostridium histolyticum* group but this finding was not shown in the vessels consisting of the ZnC components. The vessels containing WD showed a greater alteration in bacterial metabolism compared to the PHGG vessels. Optimising the dose and substrate availability is important to ensure homeostasis and prevent potential pathogenic bacterial growth.

5.2 Introduction

Zinc carnosine (ZnC) is made up of a zinc ion and L-carnosine, which is a dipeptide of β-alanine and L-histidine (Ohkawara et al., 2006). In Japan, ZnC has been used as an stomach antiulcer drug since 1995. Many human clinical trials have shown no adverse events and found toxicity to be very low (Matsukura and Tanaka, 2000). Zinc is an essential trace metal element which can easily be excreted and only around 2-3g of zinc is present in the body with about 15mg required to be consumed each day (Matsukura and Tanaka, 2000). In a rodent study, after consumption of ZnC, 13.3% of L-carnosine and 85% zinc was recovered in faeces showing that the components reached the colon and ~11% of zinc and ~50% of L-carnosine was absorbed (Sano et al., 1991). L-carnosine has been shown to be a good carrier for zinc (Matsukura and Tanaka, 2000).

Research by Sakae and Yanagisawa (2014) found that 8 out of 14 patients with stage II-IV pressure ulcers who consumed 150mg of ZnC per day were healed after 8 weeks and the average Pressure Ulcer Scale for Healing (PUSH) scores significantly improved within a week. *Helicobacter pylori* is a bacterial infection, which can lead to the development of stomach ulcers. After therapy (the usual treatment for *H. pylori* consisting of omeprazole 20mg, amoxicillin 1g and clarithromycin 500mg) the addition of ZnC at a dose of 75mg or 150mg increased the rate of eradication compared to the triple therapy alone (Green et al., 2017).

Another disease which has symptoms of ulcers is inflammatory bowel disease (IBD) an autoimmune and relapsing condition, resulting in chronic inflammation of the GI tract, it encompasses of Ulcerative Colitis (UC) (inflammation of the colon) and Crohn's disease (inflammation along the entire GI tract) (Ghouri et al., 2014). Zinc carnosine (ZnC) has been suggested as a therapeutic treatment for UC showing significant improvement in the rectum, sigmoid colon and descending colon after 1 week of being added to existing treatment plans

(Itagaki et al., 2014). Additionally, there were significantly greater clinical outcomes or remission in the ZnC group (71%) compared to the placebo (10%).

Furthermore, supplementation with ZnC increased tight junction formation and stability in 8 healthy volunteers who undertook a bout of strenuous exercise to elevate core body temperature (Davison et al., 2016). During extreme exercise performances, there is a possible increase in gut permeability allowing entry of toxins which can lead to gut symptoms and heat stroke (Davison et al., 2016). Likewise, ZnC (37.5mg twice a day) showed maintenance of gut integrity in healthy volunteers after 5 days of consumption compared to indomethacin (a nonsteroidal anti-inflammatory drug) (Mahmood et al., 2007).

The toxicity of ZnC has been assessed but there is no evidence of the impact of ZnC on the gut microbiota. The gut microbiota contains trillions of microbes and one of the main approaches for its modulation is through dietary interventions with dietary fibre, metabolism of which produces short chain fatty acids (end-points produced after fibre fermentation) which have been associated with positive health benefits to the host (Makki et al., 2018).

The aim of this study is to assess whether ZnC combined with dietary fibre (WD and PHGG) alter the bacterial metabolism compared to dietary fibre alone and/or ZnC alone. Our secondary aim is to assess the components of ZnC (zinc and L-carnosine) and their impact on bacterial metabolism. This is important as some GI disorders may be alleviated by supplementation with ZnC.

5.3 Materials and Methods

5.3.1 Subjects

Three healthy volunteers (28±4 years old) donated faecal samples. The volunteers had no history of gastrointestinal disorders, had not consumed antibiotics in the previous 3 months or prebiotic/probiotic supplements in the prior 2 weeks.

5.3.2 Basal media

Autoclaved basal medium (Peptone water 2g, yeast extract 2g, NaCl 0.1g, K_2HPO_4 0.04g, KH_2PO_4 0.04g, $MgSO_4.7H_20$ 0.01g, $CaCl_2.6H_20$ 0.01g, $NaHCO_3$ 2g, Tween 80 2ml, haemin 0.05g, vitamin K 10µl, L-cysteine HCL 0.5g and bile salt 0.5g per litre) (Sigma, St. Louis, MO) was added to each vessel (135ml per vessel) and incubated overnight in anaerobic conditions (oxygen-free nitrogen sparged at a rate of 15ml/min).

5.3.3 Faecal sample

In the morning, participants brought a fresh faecal sample (<3 hours) to the laboratory in anaerobic jars (<1% O_2 and 9-13% CO_2) (AnaeroJarTM 2.5 L and AnaeroGemTM, Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK). Samples were diluted with phosphate buffer saline (PBS) 10% (w/v) (pH 7.4) and homogenised (Stomacher 400, Seward, West Sussex, UK) for 2 minutes at 240 paddle beats per minute. Each vessel was inoculated with 15ml of faecal slurry (total working volume of 300ml).

5.3.4 Substrates

In the first fermentation run, vessels comprised of V1 blank, V2 WD (3.6g), V3 WD (3.6g) and ZnC (45mg), V4 WD (3.6g) and L-carnosine (35.1mg), V5 ZnC (45mg), V6 WD (3.6g) and zinc sulphate (9.9mg) and V7 inulin (3.6g) and ZnC (45mg). The second batch cultures consisted of V1 blank, V2 PHGG (3.6g), V3 PHGG (3.6g) and ZnC (45mg), V4 PHGG (3.6g) and L-carnosine (35.1mg), V5 ZnC (45mg), V6 PHGG (3.6g) and zinc sulphate (9.9mg) and V7 inulin (3.6g) and ZnC (45mg).

Wheat dextrin (Benefiber, GSK, Warren, New Jersey, USA) is a resistant dextrin with a DP between 12-25 (Noack et al., 2013). Partially hydrolysed guar gum (Resource Optifiber, Nestlé Health Science, London, UK) comprises of galactose (α-1-6 bonds) and mannose units

(β-1-4 bonds) with a molecular weight of 1,000 to 100,000 Da (average 20,000 Da) (Noack et al., 2013, Yoon et al., 2008). Inulin has an average DP of 12 and is made up of fructose joined by ß-(2-1) linkages (Roberfroid, 2005). Zinc carnosine has the tradename PepZin GI (Hamari Chemicals Ltd, Japan). L-carnosine (L-carnosine 98%, ACROS Organic) and zinc sulphate (Zinc sulphide 99.99% (trace metal basis) ACROS organic) were purchased from Fisher Scientific (Fisher Scientific, Leicestershire, UK).

5.3.5 Vessel conditions

A water bath was used to ensure vessels remained at body temperature (37^{o}C) and a pH meter controlled pH between 6.7-6.9. Vessels were continuously stirred throughout the experiment.

5.3.6 Samples

A 750µl sample was collected from each vessel at timepoints 0, 8, 24, 48 and 72 hours. The samples were centrifuged at 11,337 × g for 5 minutes. The subsequent pellet was used for bacterial enumeration through fluorescence *in situ* hybridisation flow-cytometry (FISH-FCM) and 500µl of supernatant used to assess organic acid production via gas chromatography. The method used to analyse FISH-FCM and organic acid production were reported in a study by Wang et al. (2019). Bacterial probes used can be found in Table 5.1.

Table 5.1. Bacterial probes names and DNA sequence used to detect common gut bacterial groups with validation references.

Probe name	Sequence (5' to 3')	Target group	References
Non Eub	ACTCCTACGGGAGGCAGC	(Wallner et al., 1993)	(Wallner et al., 1993)
Eub338 I	GCT GCC TCC CGT AGG AGT	Most bacteria	(Daims et al., 1999)
Eub338 II	GCA GCC ACC CGT AGG TGT	Planctomycetales	(Daims et al., 1999)
Eub338 III	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	(Daims et al., 1999)
Bif164	CAT CCG GCA TTA CCA CCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*	(Langendijk et al., 1995)
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis;* all *Vagococcus, Pediococcus* and *Paralactobacillus* spp., *Melisococcus, Tetragenococcus, Catellicoccus, Enterococcus*	(Harmsen et al., 1999)
Bac303	CCA ATG TGG GGG ACC TT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	(Manz et al., 1996)
Erec482	GCT TCT TAG TCA RGT ACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* clusters XIVa and XIVb)	(Manz et al., 1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group (*Clostridium* clusters I and II)	(Franks et al., 1998)
Rrec584	TCA GAC TTG CCG YAC CGC	*Roseburia* subcluster	(Franks et al., 1998)
Prop853	ATT GCG TTA ACT CCG GCAC	Clostridial cluster IX	(Walker et al., 2005)
Ato291	GGT CGG TCT CTC AAC CC	*Atopobium, Colinsella, Olsenella* and *Eggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium* and *Mycoplasma elephantis*	(Harmsen et al., 2000)
Fprau655	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	(Hold et al., 2003)
DSV687	TAC GGA TTT CAC TCC T	Most Desulfovibrionales and many Desulfuromonales	(Devereux et al., 1992)

5.3.7 Statistical analysis

All statistical results were analysed in Prism 8 version 8.4.0 (GraphPad Prism 8, San Diego, California, USA). The FISH-FCM and organic acid production were analysed using two-way mixed ANOVA to compare different test substrates and time points. Where significant differences were found, a post hoc analysis was performed using Tukey multiple comparison tests. Statistical analysis was accepted at $P < 0.05$ for all analyses.

5.4 Results

5.4.1 Bacterial enumeration

Overall, the addition of ZnC to WD enhanced bacteria metabolism to a greater extent than in the PHGG. Total bacteria counts were elevated in all intervention test vessels, except ZnC vessels, until 48h in the WD experiment (Figure 5.1G.). There was a significant increase in total bacteria counts at 24h in the WD and L-carnosine vessels ($P = 0.0402$) with a 0.85 $\log_{10}$ cells/mL increase. Similar 0.85-0.99 $\log_{10}$ cells/mL increase was found at 48h in the WD vessels ($P = 0.0410$) and WD and ZnC vessels ($P = 0.0106$), respectively. The WD vessels had significant higher total bacterial counts compared to the blank and ZnC vessels at 48h ($P \leq 0.0356$) and 72h ($P \leq 0.0410$).

The WD vessels alone lead to a significant increase in *Bifidobacterium* spp., at 8h compared to baseline ($P = 0.0124$) whereas, the WD combined with ZnC resulted in a prolonged significant increase until the end of the experiment ($P \leq 0.0473$) (Figure 5.1A). The WD and ZnC vessels increased the production of *Bifidobacterium* spp., by 0.5 $\log_{10}$ cells/mL greater than that in the WD vessels alone. The significant increase in *Bifidobacterium* spp., was also shown when compared to the blank vessels ($P = 0.0063$) and ZnC vessels ($P = 0.0070$). *Bifidobacterium* spp. also significantly increased in the PHGG and ZnC vessels ($P = 0.0405$) at 24h compared to baseline with a 1.14 $\log_{10}$ cells/mL (Figure 5.2E). The inulin and ZnC vessels was significantly higher in *Bifidobacterium* spp., at 24h compared to baseline ($P = 0.0009$), the blank ($P = 0.0130$) and ZnC vessels ($P = 0.0237$).

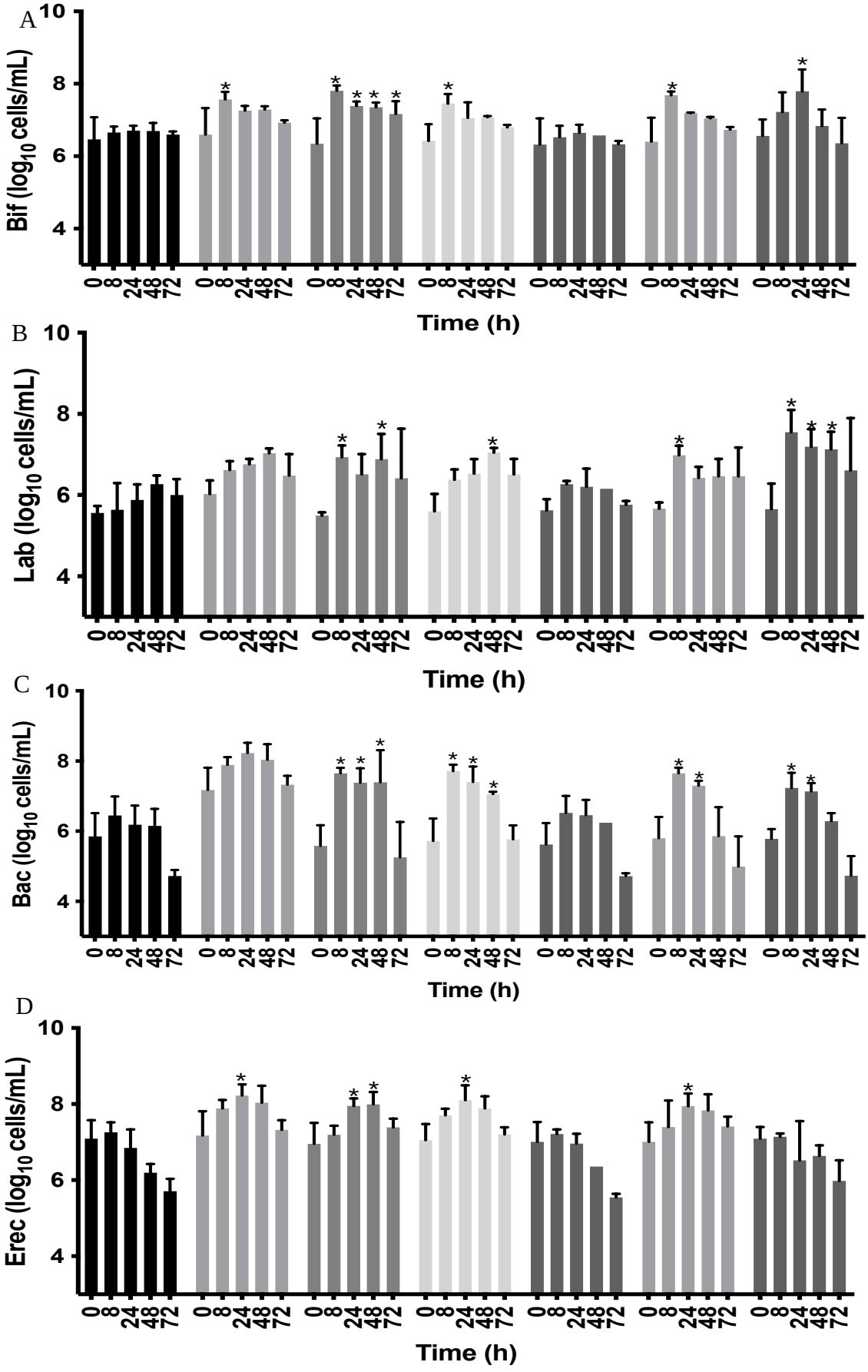
Lactic acid bacteria were not found to be significant in the WD vessels (Figure 5.1B). When WD was combined with either ZnC, zinc or L-carnosine a significant increase in lactic acid bacteria was observed. L-carnosine showed a significant increase in lactic acid bacteria early in the fermentation at 8h increasing by 1.33 $\log_{10}$ cells/mL whereas, vessels containing zinc alone did not significantly increase until 48h but showed a similar growth (1.45 $\log_{10}$

cells/mL). The inulin and ZnC vessels significantly increased lactic acid bacteria compared to baseline at 8, 24 and 48h ($P \leq 0.0018$) and compared to blank at 8 and 24h ($P \leq 0.0242$).

In the WD vessels alone no significant elevation in bacteroides was found but when combined with ZnC ($P \leq 0.0007$), zinc ($P \leq 0.0070$) and L-carnosine ($P \leq 0.0206$) a significant increase was present (Figure 5.1C). The increase in bacteroides from baseline was greater in the WD and ZnC vessels (2.06 $\log_{10}$ cells/mL) compared to the WD and L-carnosine (1.99 $\log_{10}$ cells/mL) and WD and zinc (1.85 $\log_{10}$ cells/mL) vessels.

Clostridium histolyticum group significantly increased in the WD and ZnC ($P = 0.0018$) vessels by 1.5 $\log_{10}$ cells/mL (Figure 5.1F). There was also a significant increase ($P = 0.0059$) in the inulin and ZnC vessels at 24h with an increase of 1.36 $\log_{10}$ cells/mL.

Clostridium coccoides-Eubacterium rectale group and *Clostridial cluster* IX significantly increased in the WD alone and in the WD combined with ZnC, zinc and L-carnosine (Figure 5.1D&E).


A
Bif (log10 cells/mL)
B
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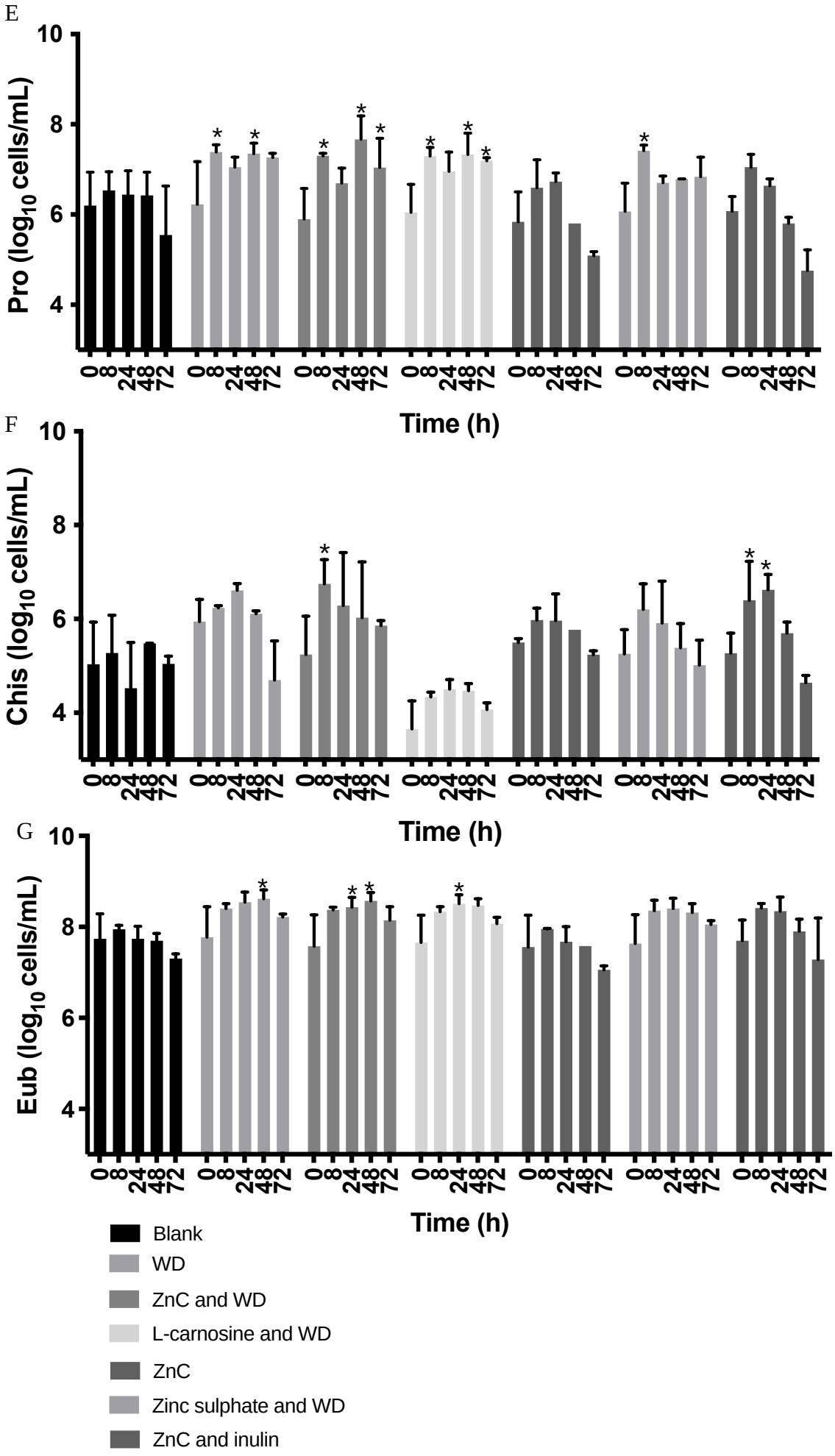
C
Bac (log10 cells/mL)
D
Erec (log10 cells/mL)
Time (h)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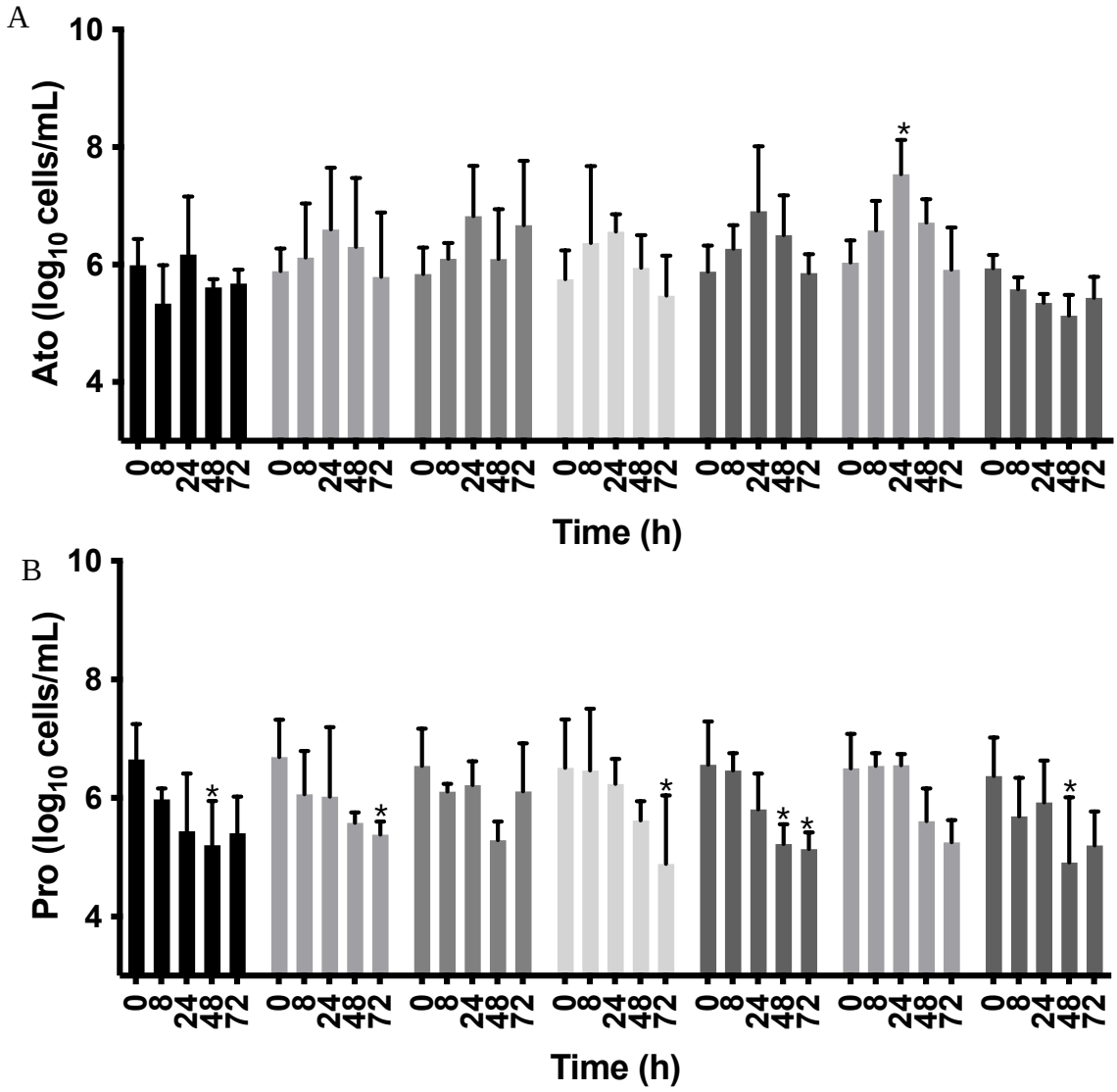
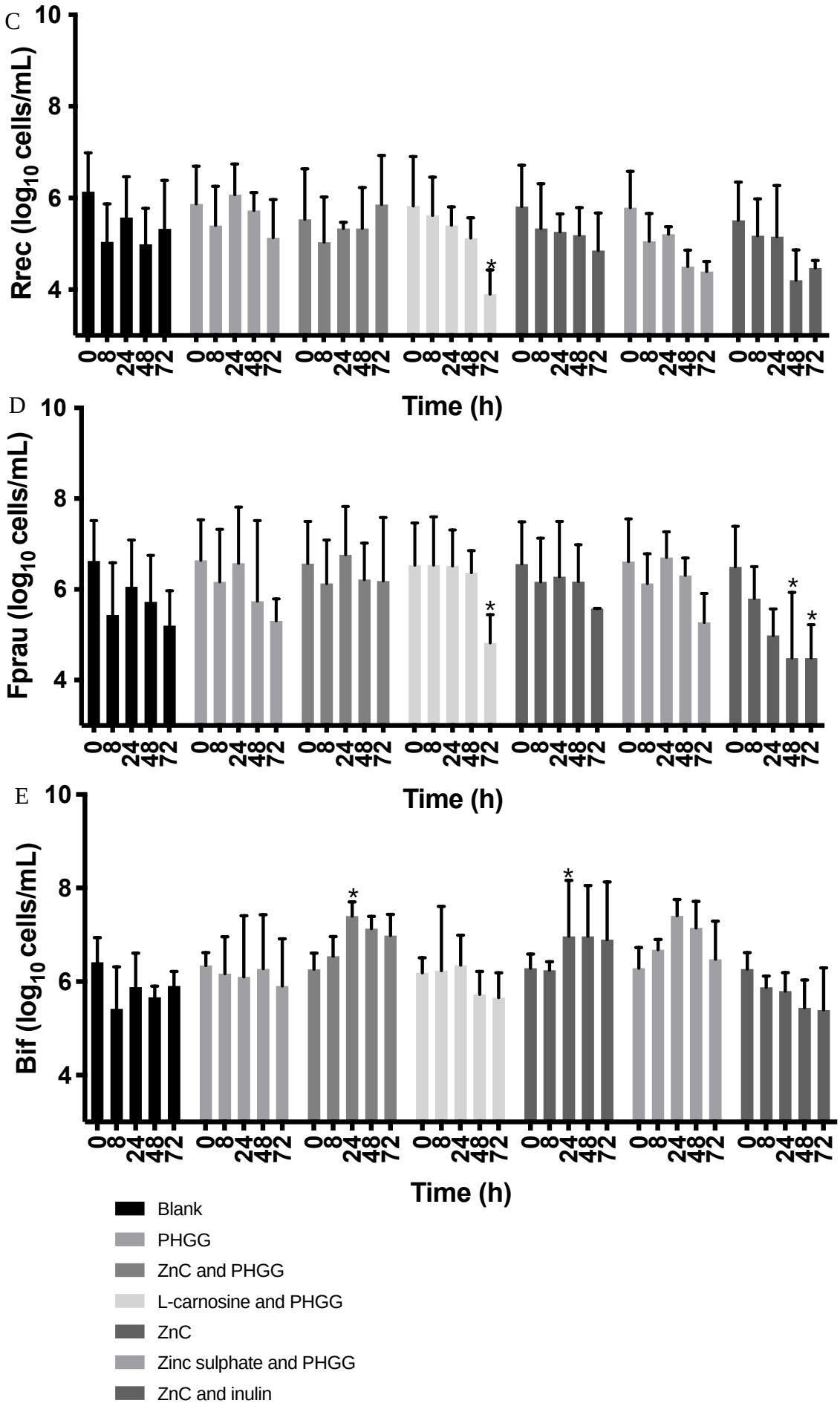
Blank
WD
ZnC and WD
L-carnosine and WD
ZnC
Zinc sulphate and WD
ZnC and inulin

Figure 5.1.

Bacterial enumeration (log_{10} cells/mL) from *in vitro* batch culture after fermenting for 0, 8, 24, 48 and 72h. The bacteria analysed were *Bifidobacterium* spp. (Bif)(A), lactic acid bacteria (Lab)(B), *Bacteroidaceae* and *Prevotellaceae* (Bac)(C), Clostridial cluster XIVa and XIVb (Erec)(D), Clostridial cluster (Pro)(E) and *Clostridium histolyticum* group (Chis)(F) and total bacteria (Eub)(G). The vessels consisted of blank, wheat dextrin (WD), zinc carnosine (ZnC) and wheat dextrin (WD), L-carnosine and wheat dextrin (WD), zinc carnosine (ZnC), zinc sulfate and wheat dextrin (WD) and zinc carnosine (ZnC) and inulin vessels. Data are presented as mean ± SD (n=3) and *shows significant difference ($P \leq 0.05$) compared to the baseline (T0).

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Bif (log10 cells/mL)
Time (h)
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PHGG
ZnC and PHGG
L-carnosine and PHGG
ZnC
Zinc sulphate and PHGG
ZnC and inulin

Figure 5.2. Bacterial enumeration (log_{10} cells/mL) from *in vitro* batch culture after fermenting for 0, 8, 24, 48 and 72h. The bacteria analysed were *Atopobium* spp. (Ato)(A), Clostridial cluster (Pro)(B), *Roseburia* (Rrec)(C), *Faecalibacterium prausnitzii* (Fprau)(D) and *Bifidobacterium* spp., (Bif)(E). The vessels were blank, partially hydrolysed guar gum (PHGG), zinc carnosine (ZnC) and partially hydrolysed guar gum (PHGG), L-carnosine and partially hydrolysed guar gum (PHGG), zinc carnosine (ZnC), zinc sulfate and partially hydrolysed guar gum (PHGG) and zinc carnosine (ZnC) and inulin. Data are presented as mean ± SD (n=3) and * shows significant difference ($P \leq 0.05$) compared to the baseline (T0).

5.4.2 Organic acid production

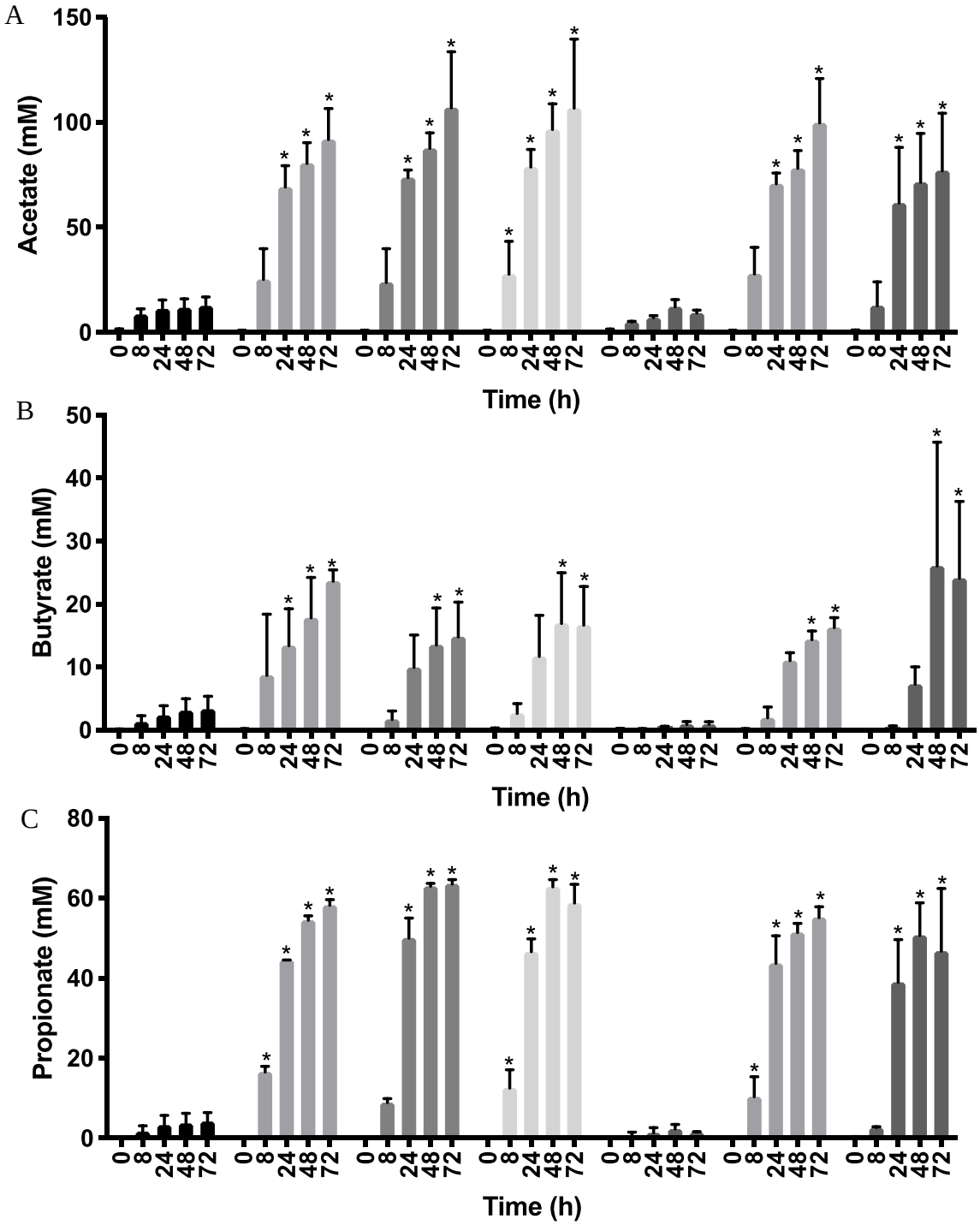
Overall, the production of acetate, butyrate and propionate significantly increased throughout the duration of the fermentation (Figure 5.3.).

Acetate production significantly increased in all intervention test vessels, except ZnC vessels, at 24h ($P > 0.0001$), 48h ($P > 0.0001$) and 72h ($P > 0.0001$) compared to baseline in the WD experiment (Figure 5.3A). The WD and ZnC vessels had the largest acetate production with an increase from baseline of 106.24mM. In the PHGG experiment acetate was significantly elevated at 72h compared to baseline in all other intervention test vessels, except for the ZnC vessels (Figure 5.3D).

Butyrate production significantly increased in all of the intervention test vessels in both the WD and PHGG experiments, except for the ZnC alone vessels and the PHGG and ZnC vessel (Figure 5.3B and 5.3E). In both experiments the vessel containing inulin showed the greatest increase in butyrate; the PHGG and inulin vessels increased in butyrate production by 51.93mM and the WD and inulin increased by 25.81mM.

Propionate was significantly elevated at 24, 48 and 72h in all of the intervention test vessels, except ZnC vessels, compared to baseline in the WD experiment (Figure 5.3C). There was a similar increase in propionate vessels ranging from an increase of 46.46 to 63.33mM in the WD experiment. In the PHGG experiment all intervention vessels increased in propionate

levels at 48 and 72h compared to baseline, except for the ZnC and PHGG and ZnC vessels (Figure 5.3F).



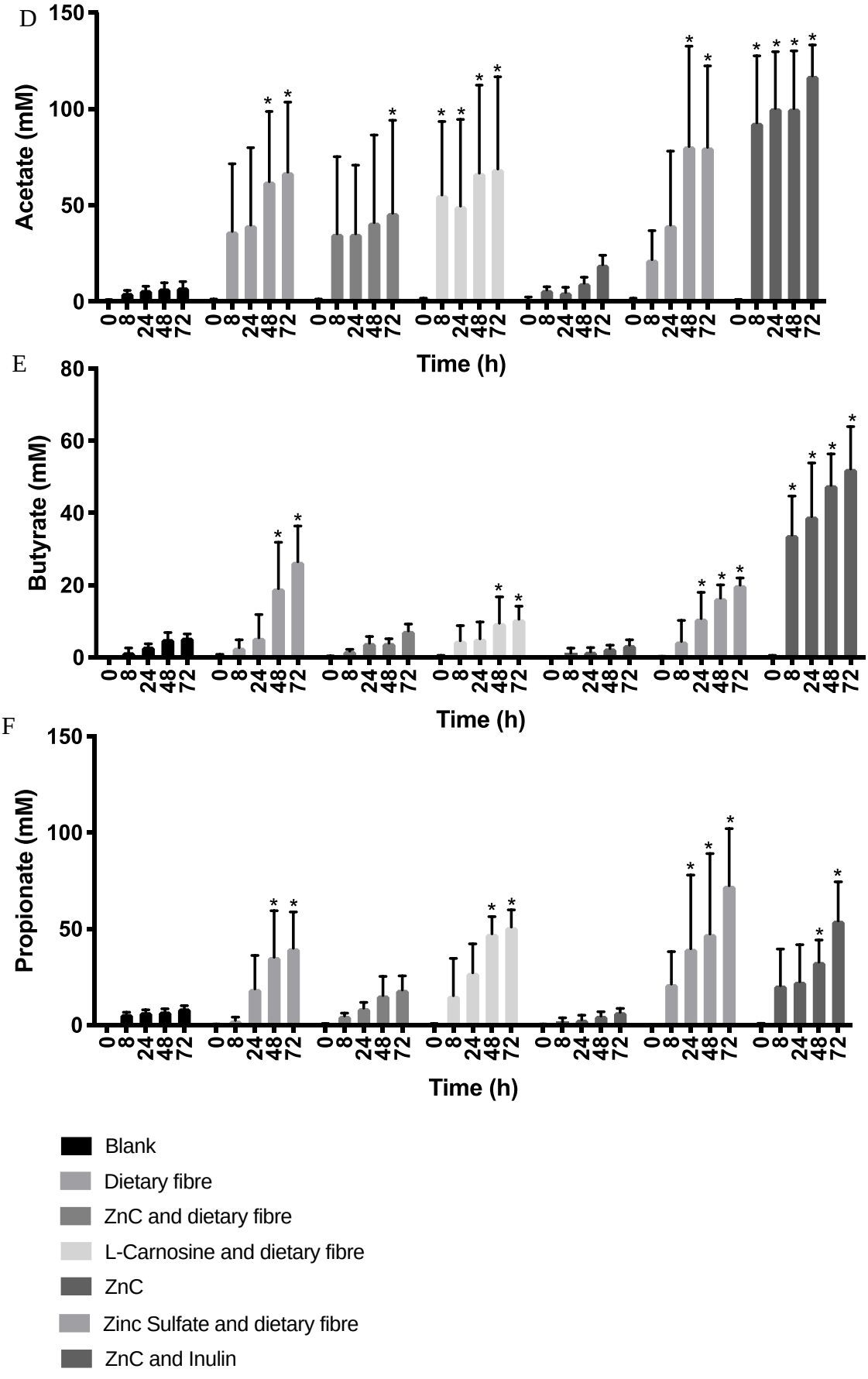

D
Acetate (mM)
Time (h)
E
Butyrate (mM)
Time (h)
F
Propionate (mM)
Time (h)
Blank
Dietary fibre
ZnC and dietary fibre
L-Carnosine and dietary fibre
ZnC
Zinc Sulfate and dietary fibre
ZnC and Inulin

Figure 5.3. Organic acid production (mM) from *in vitro* batch culture fermentation figure A, B and C shows (left hand side) the blank, wheat dextrin, zinc carnosine and wheat dextrin, L-carnosine and wheat dextrin, zinc carnosine, zinc sulfate and wheat dextrin and zinc carnosine and inulin vessels. The figure D, E and F consists of (right hand side) blank, partially hydrolysed guar gum, zinc carnosine and partially hydrolysed guar gum, L-carnosine and partially hydrolysed guar gum, zinc carnosine, zinc sulfate and partially hydrolysed guar gum and zinc carnosine and inulin. All measures were taken at 0, 8, 24, 48 and 72h. Organic acids measured were acetate, butyrate and propionate. Data are presented as mean ± SD (n=3) and * shows significant difference ($P \leq 0.05$) compared to the baseline (T0).

5.5 Discussion

Overall, the main findings were that the ZnC supplementation combined with WD prolonged the increase in *Bifidobacterium* spp., throughout the duration of the study but this did not occur in the L-carnosine and zinc vessels. Vessels containing WD showed a greater alteration in bacterial metabolism compared to the PHGG vessels.

Regulation of zinc is important as it can be toxic to bacteria if present in excess concentrations and on the other hand zinc deficiency has been associated with infectious diseases and IBD therefore zinc may be essential for the immune system and preventing inflammation (McDevitt et al., 2011, Siva et al., 2017, Suryawti, 2018).

Zinc has many functions one of which is to help maintain intestinal barrier integrity; alteration in gut barrier function allows permeation of detrimental microorganisms, antigens and proinflammatory factors. In a study using Caco-2 cells, depletion of zinc led to proteolysis of occludin which is a key tight junction protein (Miyoshi et al., 2016, Assimakopoulos et al., 2018). When intake of ZnC was consumed, intestinal barrier function was maintained or improved in healthy volunteers under high stress or when diarrhoea was induced (Davison et al., 2016, Mahmood et al., 2007). Improvements in barrier function may be important for diseases like IBD which have previously been shown to improve in terms of UC clinical outcomes and remission following ZnC supplementation (150mg/day for 1 week) (Itagaki et al., 2014).

To date, and to knowledge, this is the first study to investigate the effects of ZnC supplementation on the human gut microbiota. In the few studies which are available that have investigated the impacts of zinc, the gut microbiota has been assessed mainly by measuring the growth of pathogenic bacteria in pure culture. For example, when bacterial infection and inflammation occur there is an elevation in zinc concentrations. *Campylobacter* and *Escherichia coli* have metalloregulatory proteins, i.e. zinc uptake regulator (Zur), which regulates gene expression of znu*ABC* dimming the intake of zinc, therefore showing survival in zinc deprived environments (Davis et al., 2009, Velasco et al., 2018, Patzer and Hantke, 2000).

Some lactic acid bacteria, especially *Lactobacillus acidophilus* WC 0203, have been found to uptake zinc (Leonardi et al., 2013). In the current study, *Bifidobacterium* spp., were found to be significantly increased in the vessels containing WD and ZnC and PHGG and ZnC at various times compared to baseline with the WD and ZnC vessels showing a significant increase in *Bifidobacterium* spp., until the end of the experiment. *Lactobacillus* spp. was also significantly elevated in the WD and ZnC vessels compared to baseline. *Bifidobacterium* spp., and *Lactobacillus* spp. are considered as health-positive bacteria as shown by reducing acute diarrhoea and improve immunity in elderly participants along with other health outcomes such as decreasing inflammatory status (Gill, 2001a, Gill, 2001b, Simakachorn et al., 2000).

Plant-based foods such as nuts, grains and pulses contain phytate. Phytate binds strongly to zinc ions inhibiting absorption leading to an increase in excretion (Lönnerdal, 2000). When zinc is combined with amino acids such as histidine it increases zinc bioavailability (Lönnerdal, 2000). Absorption by bacteria is dependent on pH, temperature and microorganism species (Mrvčić et al., 2009).

However, there was a significant increase in this study, with a large standard deviation present, in the *Clostridium histolyticum* group (*Clostridium* cluster I and II) in the WD and

ZnC vessels and inulin and ZnC vessels. *Clostridium histolyticum* includes some pathogenic bacteria that can produce endotoxins and have previously been associated with disease states such as UC (Kleessen et al., 2002). The bacterial group can produce collagenase and proteinases which degrades tissues (Zia et al., 2017).

L-carnosine, found in skeletal muscle, kidney and the brain, is a good carrier for zinc and has been shown to play a role in wound healing but may also be important to improve neurodegenerative and neurological disorders (Berezhnoy et al., 2019, Matsukura and Tanaka, 2000). Cerebral ischemia, which leads to severe brain damage, may be improved by carnosine in early stage recovery as an increase in expression of glutamate via EAAC1, leads to GABA synthesis, increasing extracellular GABA as well as increasing recovery in mitochondrial energy metabolism (Ouyang et al., 2016). Research is emerging assessing the link between the gut and brain, named the gut-brain axis, and the supplementation of ZnC may be an interesting concept to investigate to gain understanding into whether ingesting ZnC could lead to alterations in neurotransmitters.

Previous research has shown an association between zinc intake and depression. A randomised, double-blind, placebo-control found, in 44 participants with major depression, that combining zinc supplementation (25mg per day for 12 weeks) with antidepressants may help to reduce severity of symptoms (Ranjbar et al., 2013, Amani et al., 2010). This finding is supported by a recent study conducted by Nakamura et al. (2019) who showed from an epidemiological study in Japan (>2000 participants) an inverse association between zinc intake and anxiety and depression. Supplementation of ZnC may be a novel treatment to target gut barrier function as well as neural aspects associated with GI disorders such as Irritable Bowel Syndrome (IBS), (Hellstrom and Benno, 2019).

5.6 Conclusion

Homeostasis of zinc is important and further research is warranted to investigate the addition of ZnC on the gut microbiota and assess the optimum dosage to elevate health benefits *in vivo*. Additionally, adding probiotic strains may diminish pathogenic bacteria. Interestingly, components of ZnC separately have been found to help neurodegenerative, neurological and psychiatric disorders therefore further research investigating the gut-brain axis could be of interest (Schon et al., 2019, Szewczyk, 2013).

5.7 References

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6 Chapter 6: General discussion and future perspectives

In the UK around half of the population have experienced some type of gastrointestinal discomfort (YouGov, 2010). One in four individuals meet the criteria for functional gastrointestinal (GI) disorders in United Kingdom, United States and Canada (Palsson et al., 2020). Diet is the key driver that alters the gut microbiota composition and bacterial metabolism (Leeming et al., 2019). Fibre has been shown to be beneficial for the gut microbiota as saccharolytic fermentation leads to favourable end products such as short-chain fatty acids (SCFA). The type and structure of fibre is important as they will be utilised by different bacteria. In Chapter 2 metabolism of varies dietary fibres was conducted using an *in vitro* batch culture system and it was found that inulin was utilised by *Bifidobacterium* spp., whereas partially hydrolysed guar gum (PHGG) and wheat dextrin (WD) were metabolised by *Bacteroides* and *Clostridium* cluster IX with PHGG been fermented to a greater extent. There was a significant increase in butyrate in the inulin vessels while, propionate was significantly elevated with WD and PHGG compared to inulin.

Following this, a synbiotic study was conducted with the aim to investigate whether there was an alteration in bacterial metabolism compared to the dietary fibre or probiotics alone. The main conclusion from Chapter 3 was that the synbiotic vessels containing inulin and probiotic strains (either *Bifidobacterium lactis* HN019 or *Lactobacillus acidophilus* NCFM) enhanced bacterial metabolism as *Bifidobacterium* spp., significantly increased for a prolonged period of time compared to inulin alone. No changes in bacterial metabolism were found in WD and PHGG vessels with either probiotic combination therefore these were not investigated further.

Irritable Bowel Syndrome (IBS), affects around 11% of people globally, it has been shown to be a good model for common GI issues such as diarrhoea, constipation, increased gut sensitivity and problems with digestion (Dupont, 2014, Ringel et al., 2011, Canavan et al.,

2014). Previously, a limited amount of studies have investigated the use of synbiotics as a treatment for IBS. A review by Gracie and Ford (2015) showed that three out of four randomised control trials improved abdominal pain, bloating and caused increases in beneficial bacteria (Min et al., 2012, Rogha et al., 2014, Tsuchiya et al., 2004). In children, a study was shown to improve IBS symptoms during synbiotic use (*B. lactis* B94 dose 5×10^9 CFU and inulin dose 0.9g twice a day) compared to the prebiotic alone (Basturk et al., 2016). However, to date no studies have used the same synbiotic combination as in this thesis (*L. acidophilus* NCFM and inulin or *B. lactis* HN019 and inulin).

Bifidobacterium spp., and *Lactobacillus* spp., may be reduced in individuals who suffer from IBS-D (Irritable Bowel Syndrome predominantly Diarrhoea) (Malinen et al., 2005, Carroll et al., 2012). In this thesis, Chapter 4 highlighted no differences in baseline bacterial communities between healthy and IBS-D sufferers but there was significantly higher propionate production in IBS-D sufferers. This has previously been supported (Tana et al., 2010, Valeur et al., 2016, Farup et al., 2016). The Tana et al. (2010) study associated higher propionate levels with more severe gastrointestinal symptoms, lower quality of life and negative emotions compared to those who had lower propionate levels. Modulation of the gut microbiota can occur through the use of probiotics which have been shown to improve IBS symptoms (Lyra et al., 2016). The modulation may also alter the gut brain interaction as certain probiotic *Lactobacillus* and *Bifidobacterium* strains have the ability to produce gamma-aminobutyric acid (GABA) (Barrett et al., 2012). Co-morbidities of anxiety and depression have been found to be associated with IBS, especially IBS-D (Banerjee et al., 2017, Bravo et al., 2011, Lee et al., 2017). In Chapter 4, it was found that prebiotic use increased GABA production, the main inhibitory neurotransmitter of the central nervous system (CNS). The synbiotic further increased production of GABA in healthy volunteers but this was not shown

in IBS-D sufferers. Overall, the prebiotic intervention had a profound effect upon bacterial metabolism.

The effect of micronutrients on the gut microbiota have been infrequently studied and to date no data has shown the impact of zinc carnosine (ZnC) on the gut microbiota. Previous studies have shown positive impacts of ZnC in Ulcerative Colitis patients (Ghouri et al., 2014, Itagaki et al., 2014). Chapter 5 showed that *Bifidobacterium* spp., was increased when ZnC was combined with either PHGG and WD. The ZnC and WD vessel also increased in lactic acid bacteria. Some bacterium in the *Clostridium histolyticum* group are pathogenic bacteria was also found to increase. Homeostasis of ZnC is important to ensure promotion of beneficial bacteria and diminish pathogens.

Overall, prebiotics seem to have major impacts on the gut microbiota but their various structures alter bacterial metabolism in different ways. Synbiotics can be an effective method to increase specific probiotic strains for their functional benefits but also have a large impact on bacterial metabolism through their incorporation with prebiotics. To date, few studies have used synbiotics with one probiotic strain but more thought needs to go into multiple use for functional benefits, cross feeding and survival. Synbiotic intervention had a positive impact on bacterial metabolism in both healthy and IBS-D donors. This supplementation also enhanced the neurotransmitter GABA in healthy donors but this was not seen in IBS-D sufferers. The use of ZnC may also help to promote improvements in anxiety and depression but more research into the optimum dose and substrate combination is required to enhance beneficial bacteria.

6.1 Limitations

The donors used for the gut models had no history of gastrointestinal disorders and had not consumed antibiotics within the last 3 months or probiotics/prebiotics within the last 2 weeks. However, multiple components can influence the gut microbiota such as genetics, age, lifestyle, habitual diet and environmental factors (Goodrich et al., 2014, Yatsunenko et al., 2012). The study focus on capturing the free living individuals to represent the general population therefore did not limit the above factors which then resulted in large standard deviations within the data due to the small study number of three donors.

The gut model system is a validated model as described by Macfarlane et al. (1998) although, model systems have limitations such as the inability to demonstrate absorption and the inability of the intervention to show health benefits which is required as part of the definition for probiotic, prebiotics and synbiotics (Gibson et al., 2017, Swanson et al., 2020, Hill et al., 2014).

In chapter 4, the fluorescence *in-situ* hybridization combined with flow cytometry (FISH-FCM) technique was used to measure the enumeration of the bacteria but there was no measure for the specific strain used in the synbiotic therefore using methods such as 16S rRNA sequencing would allow for specific strains to be tested to confirm the growth in these species.

Additionally, in chapter 4 GABA was measured through liquid chromatography-mass spectrometry within the vessels showing an increase in production but data should be analysed with caution as it is still unknown whether GABA produced in the gut reaches the brain and what concentration is required in the gut to demonstrate improvements in symptoms of anxiety and depression.

6.2 Future research

6.2.1 Synbiotics

Next steps following from *in vitro* models would be to conduct a human intervention trial. The primary aim of this study would be to assess impact of synbiotic use on IBS symptoms. A secondary aim could be to assess impact of synbiotic on neurotransmitter production as well as anxiety and depression symptoms. Additionally, further research into the function of the gut microbiota communities following the synbiotic intervention is required. In general, enhanced modelling systems to understand the ecology of the bacteria to predict cross feeding and optimise the synbiotic combination for the desired health outcome is required. The mechanism behind the elevation in propionate levels and reduction in GABA concentration in the IBS-D group needs further investigation.

6.2.2 Zinc carnosine

To further investigate the effects of zinc carnosine (ZnC) on the gut microbiota a three stage gut model system would be used prior to an *in vivo* study especially since the pathogenic effects need to be further investigated and confirmed in a follow up study. The aim of the study would be to assess the impact of ZnC on the gut microbiota in a continuous culture model. The secondary aim would be to assess the impact of ZnC on neurotransmitter production. Additionally, barrier function would be assessed as well as the interaction with neurotransmitters in the gut. In general further investigation is required into the impact of micronutrients on the gut microbiota.

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