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Gut Community Response to Wheat Bran and Pinto Bean

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GUT COMMUNITY RESPONSE TO WHEAT BRAN AND PINTO BEAN

by

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There is general consensus among the scientific community that dietary fibers reduce the risk of Western diseases through their fermentation by beneficial microbial communities in the human gut. However, dietary fibers in wheat bran (WB) and pinto bean (PB) are incompletely fermented by the gut microbiota. Therefore, there is a critical need to identify gut microbial communities that can increase fermentation of dietary fibers from these foods to maximize their disease-preventing properties. The goal of this study was to identify such communities with increased capacity to ferment the dietary fibers in WB and PB. To accomplish these goals, a stepwise *in vitro* fecal fermentation strategy with a modification to select for WB- and PB-associated microbes was employed. Over the course of 96 h of fermentation, fecal microbiomes treated with WB either maintained or improved their carbohydrate utilization capability, while the carbohydrate utilization capability of fecal microbiomes treated with PB varied by microbiome. At the end of 96 h of fermentation, WB-associated microbiomes had higher relative abundances of *Bifidobacterium, Bacteroides, Prevotella* 9, *Enterococcus, Agathobacter, Roseburia, Ruminococcus* 1, *Dialister, Mitsuokella*, and *Veillonella*, while PB-associated microbiomes had higher *Bifidobacterium, Bacteroides, Prevotella* 9, *Enterococcus, [Ruminococcus] gnavus* group, *[Ruminococcus] torques* group, *Agathobacter, Lachnospiraceae* NK4A136 group, *Roseburia, Dialister, Veillonella,* and uncl_*Enterobacteriaceae* compared with a control containing only media. There were also other genera that were associated with WB or

PB substrates, but varied by microbiome. Both WB- and PB-associated communities were propiogenic in nature. These findings are important in developing effective intervention strategies to help increase dietary fiber fermentation and reduce the risk of disease.

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PREFACE

Non-digestible carbohydrates (NDC) or dietary fibers are known to be one of the major sources of energy for the microbiota. However, not all NDC are utilized by the gut microbiota. Only one-third of the NDC from whole grains are utilized. The remaining unfermented NDC stay non-beneficial and negatively impacts gut health, thus it is crucial to determine ways to improve gut microbiota's utilization of NDC. Although dry beans are rich in dietary fibers that likely feed the gut microbiota, few studies have reported composition of pinto bean associated microbiomes.

There are two main objectives within this study: (1) To identify the characteristics of microbiota that are associated with the utilization of carbohydrates from wheat bran (WB), (2) To identify the characteristics and functionalities of pinto bean (PB) associated microbiota.

This thesis consists of 3 chapters. Chapter 1 reviews the literature on fermentation of dietary fibers from WB and PB; chapters 2 and 3 describe a step-wise *in vitro* fecal fermentation strategy with a modification to select for WB- and PB-associated microbes, respectively.

CHAPTER 1 REVIEW OF LITERATURE

1.1. Introduction

Western diseases including inflammatory bowel disease, type 2 diabetes, cardiovascular diseases, and obesity have been linked to gut health. Diet can change gut microbiota composition and potentially help to modulate gut health. Dietary fiber, which includes non-digestible carbohydrates (NDC), is a primary energy source for the gut microbiomes. Fecal biomarkers such as short-chain fatty acids and gut microbial composition have been widely used to assess intestinal health. Two food substrates that are high in NDC and commonly known as healthy foods - wheat bran (WB) and pinto beans (PB) - are both under-consumed according to the Dietary Guidelines for Americans (2020-2025). However, being high in dietary fiber and other important nutrients they have potential to be promoted as foods to modulate gut health. This review will examine the nutrient profiles of both wheat bran and pinto beans, and previous intervention studies conducted with whole grains and dry beans as substrates in relation to gut health. Overall, it is evident that both wheat bran and pinto beans are potential tools in a dietary strategy to modulate gut health.

1.2. Gut health and diseases

The gut microbiota is complex, consisting ~3.9 x 10^{13} microbial cells and thousands of uncultured bacterial species that have been recently identified (Almeida et al., 2019; Sender, Fuchs & Milo, 2016). The adult human microbiota is comprised of Bacteroidetes, Firmicutes, Proteobacteria, Fusobacterium and Actinobacteria phyla with Bacteroidetes and Firmicutes present in the greatest abundance (>90%) (Human Microbiome Project Consortium, 2012; Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005).

There are many factors that influence gut microbiota composition: the different stages of life, which include infancy (Bergström et al., 2014; Hill et al., 2017), adulthood, and elderly periods (Mariat et al., 2009); antibiotic use (Dethlefsen, Huse, Sogin, & Relman, 2008; Pérez-Cobas et al., 2013); and ecological environments (Amato et al., 2016; Escobar, Klotz, Valdes, & Agudelo, 2014; Trosvik et al., 2010) and lifestyles (Bressa et al., 2017; Kulecka et al., 2020) are known to be factors which alter the gut microbial community. Additionally, studies have also shown that western diseases, which include obesity (Ley et al., 2005), diabetes (Wen et al., 2008), cardiovascular disease (Fava et al., 2005), were linked to the gut microbiota. Finally, dietary pattern has been identified as a major driver of gut microbial composition in both *in vitro* (de Paepe et al., 2019, 2020; Herter & Kendall, 1910; Leitch et al., 2007) and *in vivo* studies (Claesson et al., 2012; David et al., 2014; De Filippo et al., 2010; Wu et al., 2011); impacting short-chain fatty acids (SCFA) production (Cummings et al., 1987; Mijakovic et al., 2016; Wang et al., 2019).

Although a standardized healthy gut microbial composition has not been defined, inflammatory proteins, antimicrobial peptides, and SCFA production have been used as fecal biomarkers to assess intestinal health (Farup, Rudi, & Hestad, 2016; Huda-Faujan et al., 2020;

Langhorst et al., 2009; Pang, Leach, Katz, & Day, 2014). Cummings et al. (1987) proposed that the highest SCFA concentration found in the gut were usually at a molar ratio of approximately 60:20:20 for acetate:propionate:butyrate. Huda-Faujan et al. (2010) suggested that SCFA production played an important factor of inflammatory bowel disease (IBD) as patients with IBD have lower abundance of the SCFA acetate, propionate, and butyrate, but higher levels of lactic and pyruvic acids compared to healthy individuals. Langhorst et al. (2009) also found differences in the SCFA production and antimicrobial protein from patients with irritable bowel syndrome (IBS) compared to healthy individuals.

Many factors including age, antibiotic use, ecological environment, lifestyle, disease, and especially dietary pattern are associated with gut microbial composition. These lead to a manipulable community effect in the gut and eventually impact gut health. To reduce the risks of disease, more research on gut microbiomes is necessary to provide better insight into the complex phenotypic expression of different gut microbial communities.

1.3. Whole grains and dry beans in diet

Dietary interventions in both short- and long-term studies were shown to alter gut microbial composition (Brahma et al., 2017; Costabile et al., 2008; David et al., 2014; De Filippo et al., 2010; Walker et al., 2011; Wu et al., 2011). Diet affects nutrients that are available to the gut microbiota and the pathways of metabolism that are present. A major nutrient for the gut microbiota, non-digestible carbohydrates (NDC) from plants, resist digestion in the upper gastrointestinal tract and are metabolized by gut bacteria to SCFA (Han et al., 2018; Topping & Clifton, 2001). Unlike the human genome, gut bacteria encode for enzymes that break down the NDC (El Kaoutari et al., 2013; Henrissat, 1991).

Whole wheat is a good source of non-digestible carbohydrates which are more commonly known as dietary fiber. A whole grain kernel is comprised of three major components, bran, endosperm, and germ. The bran is the outer layer part of a kernel that is rich in dietary fiber, vitamin B, minerals, antioxidants, and phytochemicals. After *in vitro* digestion, 53% of wheat bran is NDC (Brahma et al., 2017) and the major polymer of NDC is arabinoxylan (Saulnier et al., 2007). Arabinoxylans are classified as water-unextractable arabinoxylans and water-extractable arabinoxylans. The other NDC in wheat bran include cellulose, β-glucan, and fructans. Although early studies suggested that the NDC from whole grains are poorly fermented by the gut microbiota (Van Dokkum, Pikaar, & Thissen, 1983), a recent study suggested that whole grain processing methods could impact the fermentation of NDC by increasing the accessibility of the cell wall polysaccharides (Smith, Van Haute, & Rose, 2020). The dietary fiber from wheat bran (WB) was suggested to lower the risk of colon-related diseases (Badiali et al., 1995; Schatzkin et al., 2007; Wong, Harris, & Ferguson, 2016), reduce the risk of cardiovascular disease in diabetes (Jenkins et al., 2002), and control diabetes (Jefferson & Adolphus, 2019).

Besides whole grains, dry beans are also a good source of NDC. Dry beans have a wide range of nutrients including proteins, carbohydrates, vitamins, minerals, antioxidants, antibacterial phenolic compounds, and are low in fat (Ganesan & Xu, 2017; Kalogeropoulos et al., 2010; Lyimo, Mugula, & Elias, 1992). Among all dry beans (black beans, red kidney beans, great northern beans, lima beans, navy beans, pink beans, and small red beans), pinto bean (PB) production in the United States has been the highest in the recent years, according to USDA National Agricultural Statistics Service (2021). Cooked pinto beans (dry basis) are approximately 16.8% proteins, 36.5% polysaccharides, 31.1% insoluble fiber, and 28.2 %

soluble fiber (Campos-Vega et al., 2009). Phenolic compounds found in beans have an antimicrobial effect on certain pathogens including *Listeria monocytogenes, Salmonella* Typhimurium*, Escherichia coli* O157:H7*, Helicobacter pylori, Bacillus cereus,* and *Staphylococcus aureus* (Amarowicz et al., 2008; Gan et al., 2016; Kanatt, Arjun, & Sharma, 2011; Lara-Díaz et al., 2009; Randhir, Lin, & Shetty, 2004). Pinto bean intakes were also shown to be associated with lower blood cholesterol (Anderson et al.,1990; Finley, Burrell, & Reeves, 2007) and lower total cholesterol in humans (Shutler et al., 1989; Winham, Webb, & Barr, 2008), and increased bone mineral density and trabecular thickness (Cao, Gregoire, Sheng, & Liuzzi, 2010), and lower colon adenocarcinoma and tumor multiplicity (Huges, Ganthavorn, & Wilson-Sanders, 1997) in mice.

1.4. Potential wheat bran associated microbes

Considering that different gut microbes encode different enzymes to ferment nutrient sources (Cantarel, Lombard, & Henrissat, 2012), certain microbes are expected to interact preferentially with certain foods. For instance, a diet high in animal protein was associated with *Bacteroides* enterotype while diets high in plant carbohydrates were associated with *Prevotella* enterotype (De Filippo et al., 2010; Wu et al., 2011).

Several *in vitro* studies have been conducted with wheat bran as the sole nutrient source for fecal microbiota, in order to enrich for WB-associated fecal microbiomes. These studies utilized different *in vitro* fermentation models: Leitch et al. (2007) utilized single-stage fermentor systems; while De Paepe et al. (2017) and De Paepe et al. (2020) conducted comparable batch fermentation with hungate tubes. De Paepe et al. (2018) used the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) approach, whereby the SHIME model was validated as the gastrointestinal tract, simulating the stomach or small intestine, proximal and

distal colon conditions through a modulation of residence time and pH. Nonetheless, De Paepe et al. (2019) performed subculturing of wheat bran-attached microbes with both direct plating method and enrichment method. For direct plating method, the fecal sample was directly plated onto the solid wheat bran agar plates to isolate wheat bran metabolizing species; while the enrichment method selects for wheat bran-attaching and utilizing species by subculturing the wheat bran residue during three consecutive transfers.

Since different *in vitro* methods and fecal microbiota (subjects) were used in each of the studies, different WB-associated microbes have been reported. However, even within a study, individualized microbiome responses to dietary fiber was a great challenge to identifying WB-associated communities (De Paepe et al., 2017; De Paepe et al., 2018; Kovatcheva-Datchary et al., 2015; Leitch et al., 2007). Despite inter-study differences and inter-individual variable response, most of the microbial strains enriched in previous studies were either known as plant material degraders or encode carbohydrate active enzymes (CAZymes). *Bacteroides* and *Prevotella*, which both encode multiple CAZymes (Dodd, Mackie, & Cann, 2011), were commonly enriched through *in vitro* fermentation of WB (De Paepe et al., 2017; De Paepe et al., 2018; De Paepe et al., 2020; Leitch et al., 2007). Additionally, a variety of members of either *Lachnospiraceae* or *Ruminococcaceae* were also commonly enriched (De Paepe et al., 2017; De Paepe et al., 2018; De Paepe et al., 2020; Leitch et al., 2007). Both families have been shown to be important in the fermentation of complex plant material (Biddle et al., 2013; Brulc et al., 2009; Ding et al., 2001; Flint et al., 2008).

1.5. Potential dry bean associated microbes

There is limited literature on *in vitro* fermentation of dry bean. Only one study attempted to identify PB-associated microbiomes with human fecal microbiota (Guan et al., 2020). This study conducted an *in vitro* fermentation model with different PB constituents – intact cell, slightly damaged cells, highly damaged cells, and isolated components (starch, protein, and cell wall polysaccharides), to identify the impact of different PB substrates, especially type I resistant starch, on the gut. Guan et al. (2020) found that the intact cell and cell wall polysaccharides promoted unclassified *Lachnospiraceae*; while the slightly damaged cells, highly damaged cells and starch samples increased *Blautia* and lowered *Fusobacterium* levels. Another study conducted with PB, but with a mouse feeding model also reflected an increased relative abundance of members from the *Lachnospiraceae* in the PB group (Ojo et al., 2021). *Lachnospiraceae* has been linked with dietary fiber fermentation (Biddle et al., 2013).

An *in vitro* fermentation with pinto beans and pig fecal microbiota (Chen et al., 2020) showed an increase in the saccharolytic bacteria *Prevotella copri*, *Bacteroides vulgatus,* and the succinate-utilizing bacterium *Phascolarctobacterium succinatutens* (Watanabe, Nagai, & Morotomi, 2012). Rovalino-Córdova et al. (2020) found an increased abundance of *Bifidobacterium* through an *in vitro* fermentation of red kidney beans with a dynamic *in vitro* simulator model of the human digestion system - simulator of the human intestinal microbial ecosystem (SHIME®). Similar results were also noted in another study (Teixeira-Guedes et al., 2020) conducted with *in vitro* fermentation of cowpea and black bean. The treatment group showed higher increasedof *Bifidobacterium* and *Lactobacillus,* which are well known as oligosaccharides degraders (Kailasapathy, & Chin, 2000; Shah & Lankaputhra, 1997). An enrichment of the saccharolytic bacterium *Bacteroidales* S24-7 was discovered in mouse feeding trials with either black bean, navy bean or pinto beans (Monk et al., 2017; Ojo et al., 2021;

Ormerod et al., 2016). Monk et al. (2017) have also found higher abundance of cellulolytic species, *Rumunococcus flavefaciens* and SCFA-producing microbes, *Prevotella* spp. in the treatment group (black beans and navy beans).

On the other hand, the phenolic compounds in dry beans are known to exhibit antimicrobial effect. (Amarowicz et al., 2008) showed a consistent high level (125 to 250 μg/ml) of tannin extract from red beans' phenolic compounds was effective in inhibiting *Brochothrix thermosphacta, Staphylococcus aureus, Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Pseudomonas fragi*, and *Lactobacillus plantarum*. Another study discovered a strong inhibitory effect of 0.1% and 0.2% extracts of Bengal gram (garbanzo bean) hulls on *Bacillus cereus* (Kanatt et al., 2011). Additionally, Randhir et al. (2004) proposed that the phenylpropanoid pathway was stimulated in mung bean sprouts through the pentose phosphate and shikimate pathways and elicited antibacterial activity on a *Helicobacter pylori* strain. Perla black bean extracts inhibited growth of *Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella oxytoca, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis,* and *Listeria monocytogenes* (Lara-Díaz et al., 2009). By incorporating PB into a mouse diet, Ojo et al. (2021) found positive correlation between antimicrobial peptide genes (Reg3 γ and Reg3 β) and members from *Lachnospiraceae* NK4A136 group. A similar antimicrobial component was shown in another study (Fernando et al., 2010) utilizing chickpea, where chickpea feeding reflected a higher abundance of *Faecalibacterium prausnitzii*, and lower abundance of a pathogenic bacteria species, *Clostridium histolyticum* (Fernando et al., 2010).

The overall limited literature has proposed a variety of microbes that are associated with dry beans, with a majority from saccharolytic and cellulolytic microbes. However, PB-mediated

gut metabolic reactions such as branched chain fatty acid production, and the possible inter-individual difference impact on gut microbiota, remain unknown. To provide better insight on utilizing PB as a functional food to modulate gut health, more studies are needed to identify the characteristics and functionality of pinto beans (PB) associated microbiomes.

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CHAPTER 2 CHARACTERISTICS OF WHEAT BRAN-ASSOCIATED MICROBIOTA

2.1. Abstract

Although wheat bran (WB) is rich in non-digestible carbohydrates (NDC), they are poorly fermented by the gut microbiota, limiting their health-promoting properties. To maximize the fermentation of NDC, identification of gut microbial communities that are involved in utilization of WB NDC is a crucial step to maximizing the human health benefits that arise from consumption of WB and whole grains. In this study, a stepwise *in vitro* fecal fermentation strategy with innovative modifications was employed to select for WB-associated microbes from among four human fecal microbiotas. WB-associated microbiotas maintained or improved their carbohydrate utilization capability over the course of 96 h of fermentation compared with the control (fermentation media only). The carbohydrates fermented ranged from 10.3% to 33.9%, $p<0.05$, except for one anomalous time point for microbiome 4 at 72 h. Concomitantly, there was a dramatic decrease in observed amplicon sequence variants (ASVs), but an increase in Shannon diversity index. These results indicate that my *in vitro* approach had successfully enriched for a WB NDC-utilizing microbiota. Since the fermentation steps were under high dilution pressure and only digested wheat bran was provided as the sole nutrient source in the WB group, all microbiomes converged to a more similar composition with subtle differences. Almost half of the increased relative abundance genera among the four microbiomes were from either *Lachnospiraceae* or *Ruminococcaceae* families, the potential carbohydrate degraders. The several taxa that were characterized in all microbiomes after 96 h of fermentation with WB included *Bifidobacterium, Bacteroides, Prevotella* 9, *Enterococcus, Agathobacter, Roseburia,*

Ruminococcus 1, *Dialister*, *Mitsuokella*, and *Veillonella*. This indicates that the phenotypic plasticity of the gut microbiome is often a result of community effects. All WB-associated communities were propionigenic in nature; propionate produced from 24 h to 96 h ranged from 20.9 μmol/g to 84.4 μmol/g, $p<0.05$. These findings will be useful to develop strategies in improving the fermentation of non-digestible carbohydrates.

2.2. Introduction

Intestinal health plays a crucial role in modulating the risks of Western diseases including inflammatory bowel disease, type 2 diabetes, cardiovascular diseases, and obesity (Remely et al., 2014; Ley, Turnbaugh, Klein, & Gordon, 2006; Scher et al., 2015). While these diseases are linked to an imbalanced gut microbiota (Mosca, Leclerc, & Hugot, 2016), the gut microbial diversity and composition are known to be altered by certain dietary nutrients, which influence the production of metabolites like short chain fatty acids (SCFA) and branched chain fatty acids (BCFA) (Leitch et al., 2007; Ze, Duncan, Louis, & Flint, 2012; Louis, Hold, & Flint, 2014; Machiels et al., 2014). Additionally, studies show that high dietary fiber foods such as whole grains are associated with SCFA production (Han et al., 2018; De Paepe et al., 2019). The non-digestible carbohydrates (NDC) from dietary fiber are fermented by gut microbes into SCFA as an energy source while promoting gut integrity and lower inflammation (Han et al., 2018; Tolhurst et al., 2012; Cox et al., 2009). Although several studies have attempted to enriched for wheat bran associated microbial strains, a shared challenge in these studies, the inter-individual effect on the WB-associated microbiomes, provides an opportunity for methodology improvement.

Subsequently, although increasing NDC-rich foods in the daily diet would be a practical way to improve gut health, research studies and survey statistics reflect that there are many challenges in utilizing NDC as a dietary intervention for gut health. One of the challenges is that most Americans do not consume enough dietary fiber. According to the Dietary Guidelines for Americans (2020-2025), more than 90 percent of American adults under-consumed dietary fiber, with more than 85 percent of adults under-consuming fruits, vegetables, and whole grains and more than 95 percent of toddlers under-consuming whole grains (U.S. Department of

Agriculture, U.S. Department of Health and Human Services, 2020). Despite being rich in NDC, only approximately one third of NDC from whole wheat are fermentable by the gut microbiota (Van Dokkum, Pikaar, & Thissen, 1983). In addition, due to the gut microbiome complexity and the large inter-individual variation in microbial composition, it remains a great challenge to identify and enrich for a WB NDC-metabolizing community of microbes (Salonen et al., 2014; Deehan et al., 2020).

Several studies were able to enrich for a few similar bacterial strains through *in vitro* fermentation of wheat bran. Most of these strains were either known as plant material degraders or encode carbohydrate active enzymes (CAZymes). *Bacteroides* and *Prevotella*, which both encode multiple CAZymes (Dodd, Mackie, & Cann, 2011), are commonly enriched through *in vitro* fermentation of WB (De Paepe et al., 2017; De Paepe et al., 2018; De Paepe et al., 2020; Leitch et al., 2007). Additionally, a variety of members of either *Lachnospiraceae* or *Ruminococcaceae* were also commonly enriched (De Paepe et al., 2017; De Paepe et al., 2018; De Paepe et al., 2020; Leitch et al., 2007). However, the result from these studies reflected inter-individual differences in the WB-associated microbial composition and the authors recognized that individualized microbiome responses to dietary fiber is a great challenge in identifying WB-associated communities (De Paepe et al., 2017; De Paepe et al., 2018; Kovatcheva-Datchary et al., 2015; Leitch et al., 2007). To encounter the inter-individual differences, a step-wise *in vitro* fermentation model with modification was utilized for this study. This model created a rather competitive environment for the fecal microbiomes, retaining only the essential WB utilizing strains because of the high selection pressure (1:100 dilution factor during each transfer). Additionally, “critical wash step” was included after every 24 h of fermentation period before

inoculating the next batch, in order to wash off the non-WB-associated microbes and retaining the WB-attached microbial community.

Given that gut health is related to Western diseases, research studies are crucial in closing the gap of these challenges. Considering different gut microbes encode different enzymes to ferment nutrient sources (Cantarel, Lombard, & Henrissat, 2012), a group of key microbes was proposed to utilize the NDC from whole grains. Thus, the goal of this study is to identify gut microbial communities that can increase the fermentation of dietary fibers from wheat bran. Only WB-associated microbes were expected to maximize the disease-preventing properties of this under-consumed nutrient.

2.3. Materials and Methods

2.3.1. Wheat bran processing

Wheat bran was milled from hard red winter wheat (*Triticum aestivum* L. 'Scout 66') using an approved laboratory method (AACC Approved Methods of Analysis). Then, the separated wheat bran was finely milled using a cyclone mill (UDY 3010-030 Cyclone Sample Mill, UDY Corporation Ft. Collins, Colorado) equipped with a 1 mm diameter screen and stored at -80°C until digestion.

2.3.2. Fecal samples processing for *in vitro* fermentation

All procedures involving human subjects were approved by the UNL Institutional Review Board (20160816311EP). Four volunteers donated their fecal samples after signing the consent forms indicating that they had no known gastrointestinal disease, were 19 years of age or older, had not consumed antibiotics or probiotic supplements in the last 6 months, and were not a regular consumer of yogurt. Fresh fecal samples were weighed and stomach mixed with phosphate-buffered saline containing glycerol as a cryoprotectant (8 g/L sodium chloride, 0.2 g/L potassium chloride, 1.44 g/L disodium phosphate, 0.24 g/L monopotassium phosphate, 100 mL/L glycerol, pH 7.3) in ratio 1:9 (w/v), filtered with filtra bags (Filtra-Bag, Thomas Scientific, New Jersey), and divided into 15 mL aliquots inside of an anaerobic chamber (Bactron X, Sheldon manufacturing, Cornelius, OR, USA, containing 5% H_2, 5% CO_2, and 90% N_2). Aliquots were stored at -80°C until fermentation.

2.3.3. *In vitro* digestion

In vitro digestion was performed as described (Yang, Keshavarzian, and Rose, 2013) with minor modifications. Wheat bran (25 g) was boiled in 300 mL of distilled water for 20 min with stirring. Next, 1 M HCl was added to adjust the pH to 2.5, followed by the addition of 10 mL of 1.7% (w/v) pepsin (P7000, Sigma, St Louis, MO, USA) in 50 mM HCl before incubation with orbital shaking at 150 rpm for 30 min at 37°C. Then, 50 mL of 0.1 M sodium maleate buffer (pH 6, containing 1 mM calcium choride) was added and the pH was adjusted to 6.9 with 1 M sodium bicarbonate. Fifty mL of 12.5% (w/v) pancreatin (P7545, Sigma) in sodium maleate buffer was then added followed by 2 mL of amyloglucosidase (3260 U/mL, Megazyme, Bray, Ireland) before incubation with 150 rpm orbital shaking for 6 h at 37 °C. Following digestion, the mixture was transferred to dialysis tubing (molecular weight cutoff, 3500Da) and dialyzed against distilled water at 4 °C for 4 d with a water change at least every 12 h. Then, the dialysis retentate containing the undigested material was freeze dried (FreeZone Tray Dryer, Labconco, Kansas City, MO, USA).

2.3.4. *In vitro* fermentation

Stepwise *in vitro* fecal fermentation was performed according to Kok et al. (2019) with modifications to select for WB-associated microbes (Figure 2.1). First, in a 15 mL centrifuge tube, 100 mg of the freeze dried retentate (*in vitro* digested WB) was suspended in 9.9 mL of sterile fermentation medium for 30 min. Tubes were prepared in duplicate per replication (four tubes total); one tube was used for carbohydrate quantification and the other tube was used for microbiota composition, pH measurement, and SCFA analysis. A control was also prepared that did not contain WB. Next, 0.1 mL of each of four fecal slurries processed earlier were added separately to the WB slurry or control and incubated at 37 °C with orbital shaking (300 rpm) for

24 h. The fermentation medium used in the control group contained (per L): peptone (2 g, Fisher Scientific, Waltham, MA), yeast extract (2 g, Fisher Scientific, Waltham, MA), bile salts (0.5 g, Oxoid, Cheshire, England), sodium bicarbonate (2 g), sodium chloride (0.1 g), dipotassium phosphate (0.08 g), magnesium sulfate heptahydrate (0.01 g), calcium chloride hexahydrate (0.01 g), L-cysteine hydrochloride (0.5 g, Fisher Scientific, Waltham, MA), hemin (50 mg dissolved in DMSO), Tween 80 (2 mL, Fisher Scientific, Waltham, MA), vitamin K (10 μL, dissolved in ethanol, Alfa Aesar, Haverhill, MA), and 0.025% (w/v) resazurin solution (4 mL, dissolved in water, Alfa Aesar, Haverhill, MA), prepared according to Yang, Keshavarzian, and Rose (2013). A liter of fermentation medium prepared with 2 g of peptone would contain approximately 0.0198 g of nitrogen in 9.9 mL of fermentation media. In each centrifuge tube, 100 mg of freeze dried retentate (*in vitro* digested WB) contained approximately 0.0145 g of nitrogen. To standardize the amount of nitrogen in the fermentation of the WB group and control group, only 0.67 g instead of the 2 g of peptone used for the control group was added into the 1 L fermentation medium that was used in WB group.

At 24 h, the duplicate tubes underwent a "critical wash step" to select for a WB-associated consortia, according to De Paepe *et al.* (2017) and De Paepe *et al.* (2019) with modification. First, tubes were centrifuged at 700 rpm, 5 min to pellet the WB and associated microbes, the supernatant was separated for pH measurement and SCFA analysis. Next, the pellets were washed three times with 5 mL of fresh fermentation medium using the same centrifugation conditions. After discarding about 3 mL of the supernatant (wash medium) from the last centrifugation (leaving WB-associated microbes suspended in about 2 mL of fermentation medium), one of the duplicate tubes were removed from the anaerobic chamber and immediately frozen in liquid nitrogen for future carbohydrates analysis; from the other tube 0.1

mL of WB-associated consortia suspension was transferred in duplicate into fresh centrifuge tubes containing 100 mg of freeze dried retentate and 9.9 mL of fermentation media. This mixture was fermented for another 24 h. At the end of this fermentation period, the tubes were designated as 48 h tubes and the same freezing and critical washing steps were performed as described for the 24 h tubes. This was continued for a total of 96 h of fermentation. The entire process was conducted inside an anaerobic hood, including centrifugation, washing, inoculation, and fermentation. Frozen tubes were then stored at -80°C for fermentation analyses use.

2.3.5. Fermented carbohydrates

The residues of carbohydrates that were not fermented by the microbiomes were hydrolyzed according to Englyst, Quigley, Hudson, and Cummings (1992). Tubes designated for carbohydrate analysis were centrifuged at 14,000 rpm for 5 min and the pellet was separated from supernatant and both were retained. Then, approximately 4 mL of concentrated sulfuric acid was added to the pellet to obtain a final concentration of 12 M. After vortex mixing, the mixture was incubated at 35°C for 1 h with occasional mixing. The reserved supernatant was added back to the hydrolyzed pellet and 25 mL of distilled water was used to quantitatively rinse any remaining supernatant back into the tube with the hydrolyzed pellet. This resulted in a final sulfuric acid concentration of 2 M. The mixture was then placed in a boiling water bath for 1 h. The concentration of carbohydrates in the hydrolyzed samples were then diluted in ratio 1:10 and measured by the phenol-sulfuric acid method using diluted glucose in the concentration of 0.01, 0.05, 0.1, 0.15, and 0.21 mg/mL as the standards (Dubois et al., 1956). The percentage of carbohydrates fermented was calculated based on the difference between the carbohydrates supplied at the beginning of each 24 h period and the carbohydrates remaining after fermentation

divided by the quantity at the beginning of fermentation. Carbohydrate utilization in the first 24 h of fermentation was compared with that of other time points to determine whether the microbial community changed in its ability to ferment NDC from WB.

2.3.6. Microbiota composition

DNA extraction was done with BioSprint 96 One-For-All Vet Kit (Qiagen) according to BioSprint 96 One-For-All Vet Handbook. Prior to DNA extraction, the fermented samples were thawed in ice for around 30 mins, followed by centrifugation at 14,000 rpm, 5 mins to obtain the pellets of microbes. The pellets were suspended in warmed ASL stool lysis buffer and transferred into 96 deep well bead-beating plates. Plates were then bead beaten 4 times (4 mins, 1800 rpm) with rest intervals of 10 mins, followed by 70 °C incubation in water bath for 10 mins. While bead beating, wash plates were prepared according to the manual. Then, 100 µL of samples were added into an S-block loaded with 40 µL proteinase K, incubated in water bath at 70°C for 10 mins. Finally, for 96 samples, RLT mixture containing buffer RLT (35 mL), isopropanol (35 mL), Mag Attract Suspension G (3 mL), and buffer AVE with carrier RNA (310 µL) was added into the warmed S-block (each well 600 µL of RLT mixture) before placing into BioSprint 96 for automated extraction. Extracted DNA samples were then subject to amplicon sequencing of the V4 region of the 16S rRNA gene on the Illumina MiSeq platform using MiSeq Reagent kit v2 (2 X 250 bp) following the procedure in Kozich et al. (2013). Illumina data were processed according to the following steps. Utilizing the QIIME 2 platform, the sequence analysis was performed once sequences were demultiplexed and barcodes were removed (Bolven et al., 2019). Next, DADA2 was used to perform sequence quality control, trimming, chimera removal and denoising (Callahan et al., 2016). To maintain sequence qualities above a phred

score of 30, forward and reverse reads were truncated to 245 and 160 bp. Sequences were dereplicated into 100% ASVs with DADA2 for exact sequence matching. SILVA database46 were used to assign the taxonomy (Quast, C. et al., 2013). Before statistical and diversity analyses, the reads were rarefied to a sampling depth of 9,000. QIIME2 was used to calculate the diversity metrics of the fecal and fermented samples.

2.3.7. Short-chain fatty acids

SCFAs were extracted from the supernatant of fermented samples separated after the centrifugation performed prior to DNA extraction for 16S sequencing and measured by gas chromatography as in Hartzell, Maldonado-Gómez, Hutkins, & Rose (2013). In short, 0.4 mL of supernatant from fermented samples, 0.1 ml of 7 mM 2-ethylbutyric acid in 2 M potassium hydroxide, 0.2 ml of 9 M sulfuric acid, and sodium chloride were added and vortex mixed. Then, 0.5 mL of diethyl ether was added and centrifuged at 14,000 rpm, 2 min. The separated top layer, diethyl ether phase, was collected and injected into a gas chromatograph (Clarus 580, PerkinElmer, MA USA) with capillary column (Elite-FFAP, 15 m×0.25 mm inner diameter×0.25 μm film thickness, PerkinElmer, Waltham, MA, USA) and the SCFAs were detected by a flame ionization detector at 240 °C. SCFA data were then quantified by calculating response factors for each SCFA relative to 2-ethyl butyric acid using injections of pure standards.

2.3.8. pH analysis

Supernatant from fermented consortia at 24 h, 48 h, 72 h, 96 h and supernatant of consortia mixture before fermentation at 0 h were collected for pH measurement. This supernatant was obtained after the first centrifugation step in the “critical wash step” mentioned

in the *in vitro* digestion method. Measurement was carried out using a pH probe (FiveEasy F20, Mettler Toledo, Switzerland).

2.3.9. Statistical analysis

For carbohydrate, SCFA, and α-diversity data, a two factor ANOVA was performed where subject and time were the factors. Within each subject and across all subjects, Dunnett's test was used to determine significant differences between samples using the 24 h fermented samples as control, and to determine significant differences between treatment group (wheat bran) and control group (without wheat bran). All ANOVAs were run and analyzed using SAS software (version 9.4, Cary, NC USA).

The community sequencing data was analyzed with DESeq2 package (Love & Huber & Anders, 2014) in RStudio (version 3.4.3). Features that account for the least occurrence, ASVs (<0.1% in all samples) were filtered and removed to accurately identify significant differences in taxonomic composition between the control group at 96 h and NDC utilizers at the end of fermentation, 96 h. The remaining features represented in 139 genera were used for analyses. Significant genera were calculated by Log2-fold change with a value higher than 0 and adjusted p value of <0.05.

2.4. Results

2.4.1. Carbohydrate utilization

The WB-associated microbiomes fermented 10.3% to 33.9% of the total NDC supplied during each 24 h period of fermentation, except for one anomalous time point for microbiome 4

at 72 h (Figure 2.2). Importantly, all WB-associated microbiomes either maintained (microbiomes 1, 2, and 4) or significantly improved (microbiome 3) their carbohydrate utilization capability ($p<0.05$) after 96 h of stepwise *in vitro* fermentation compared with the first 24 h.

2.4.2. Microbiota diversity

Principal coordinates analysis (PCoA) of Bray-Curtis distances among samples showed major clustering based on treatment, and treatment-induced shifts over time (Figure 2.3). Additionally, the distance between samples of all microbiomes was drawn closer as fermentation hours prolonged. In terms of observed ASVs, all four microbiomes showed a dramatic decrease in observed ASVs in the first 24 h of *in vitro* fermentation. After prolonged fermentation, observed ASVs either stayed consistent with the new low level at 24 h or decreased further (Figure 2.4). Shannon diversity also decreased during the first 24 h of fermentation; however, in contrast to observed ASVs, most microbiomes increased in Shannon diversity in subsequent time points (Figure 2.4).

2.4.3. Microbiota composition

Abundances of genera in the WB-associated microbiomes were compared with the control microbiomes containing no WB. The analysis at 96 h of fermentation showed significantly higher abundance of several genera depending on starting microbiome (Figure 2.5). At least one of the four microbiomes were characterized by members of the *Parabacteroides* (microbiome 2), *Clostridium* sensu stricto 1 (microbiome 2), *[Eubacterium] hallii* group (microbiome 3), uncl_*Lacnospiraceae* (microbiome 2), *Dorea* (microbiome 2), *Lachnospiraceae*

NK4A136 group (microbiome 4), *Butyricicoccus* (microbiome 3), *Phascolarctobacterium* (microbiome 4), and *Acinetobacter* (microbiome 3) genera. Despite inter-individual differences, there were several taxa that were characterized in all microbiomes. These taxa included members of the *Bifidobacterium, Bacteroides, Prevotella* 9, *Enterococcus, Agathobacter, Roseburia, Ruminococcus* 1, *Dialister, Mitsuokella,* and *Veillonella* genera (Figure 2.6). There was a total of 19 microbial genera associated with WB-associated microbiomes. Almost half of the total, 8 out of 19 microbial genera were from the *Lachnospiraceae* and *Ruminococcaceae*.

2.4.4. Gut metabolites and pH

Gut microbial metabolite production varied among subjects (Figures 2.7-2.10). Butyrate produced by all microbiomes treated with WB (except microbiome 2) was significantly higher ($p<0.05$) at 96 h when compared to 24 h (Figure 2.7). However, butyrate production from microbiomes 3 and 4 were significantly higher in the control group that did not contain WB, compared to the WB group ($p<0.001$). All microbiomes (except microbiome 1) treated with WB either maintained or improved ($p<0.05$) the total branched chain fatty acid, BCFA (a total of isobutyrate and isovalerate), production at 48 h, 72 h, and 96 h when compared to 24 h of fermentation. Overall, the average of BCFA production in control group was higher ($p<0.001$) compared to the treatment group (Figure 2.8 (B)).

All microbiomes either maintained or improved propionate (Figure 2.9) and acetate production (Figure 2.10) over time in the WB group. The average amount of propionate produced by all microbiomes were significantly higher at latter time points when compared to the 24 hours of fermentation ($p<0.05$). All microbiomes treated with WB had significantly higher propionate ($p<0.0001$) and acetate ($p<0.05$) production compared to control.

The pH values of all microbiomes in the WB group ranged from 6.1 ± 0.04 to 6.8 ± 0.04 and were significantly lower ($p<0.0001$) than the control group (Figure 2.11). All subjects' fermented consortia in the WB group shared the same trend. The pH values were the lowest at 24 h, then increased at 48 h, followed up by a decreasing trend from 72 h to 96 h.

2.5. Discussion

To select for WB NDC-utilizing microbiota, digested WB was fermented with fecal microbiomes from four human subjects. Every 24 h the WB-associated microbiotas were transferred to new medium containing digested wheat bran, aiming to select for microbial communities that attach to and metabolize WB NDC. Over the course of 96 h of fermentation, fecal microbiomes treated with WB either maintained or improved their carbohydrate utilization capabilities. Carbohydrate utilization was sustained despite a decrease in the observed ASVs and increase in Shannon diversity. This result suggested that the goal to enrich microbial communities that were capable of fermenting NDC from WB was achieved, while removing the non-WB-associated microbes and driving the remaining microbes to be evenly abundant.

As anticipated, across all four microbiomes, the observed ASVs in the WB group decreased over time and were significantly less than in the control group. Since only digested WB was provided to the fecal microbiomes under high dilution pressure, only WB-associated microbes were expected to persist, thus decreasing the ASV types in WB-associated microbiomes. A decrease in richness and diversity of species was also recorded in previous studies conducted *in vitro* fermentation of wheat bran (De Paepe et al., 2019; Leitch et al., 2007). Given that the fermentation capabilities and the abundance of remaining taxa in Shannon diversity index was mostly maintained over 96 h of fermentation, the higher abundant microbes

selected in microbial communities at 96 h were proposed to be either the degraders of NDC from WB or consumers of substrates broken down from NDC.

Over time, PCoA analysis based on Bray-Curtis distance showed that all fermented samples slowly converged to be more similar even between different microbiomes. This indicates that a similar group of gut microbes have been enriched in all four fecal microbiomes, as anticipated. This outcome is different from previous studies which showed that even after fermentation, microbiomes treated with wheat bran remain distinct (De Paepe et al., 2017; De Paepe et al., 2018; De Paepe et al., 2020). Although these previous studies were conducted with different *in vitro* fermentation methods, De Paepe et al. (2017) and De Paepe et al. (2020) each employed comparable batch fermentation with hungate tubes; while De Paepe et al. (2018) utilized the Simulator of the Human Intestinal Microbial Ecosystem approach, the fermentation steps did not involve a high selection pressure. Therefore, the contrasting result could be accounted by the advantage of high selection pressure (1:100 dilution factor during each transfer) from the stepwise *in vitro* fecal fermentation strategy that was utilized.

As the β-diversity result suggested, the microbiomes appeared to become more similar; likewise, the result from DESeq analysis also reflected similarity in terms of the significantly higher relative abundance of certain taxa but also some differences. In the WB groups, 19 genera were significantly more abundant than the control at 96 h of fermentation across all fecal microbiomes with inter-individual differences. Most of these genera (*Clostridium sensu stricto* 1, *[Eubacterium] hallii* group, *Lacnospiraceae*, *Dorea*, *Lachnospiraceae* NK4A136 group, and *Butyricicoccus*) belonged to either the *Lachnospiraceae* or *Ruminococcaceae* families. The result from previous studies (Leitch et al., 2017; De Paepe et al., 2017; De Paepe et al., 2018; De Paepe et al., 2020) also reflected that species from *Lachnospiraceae* or *Ruminococcaceae* were

associated with the fermentation of wheat. Both families have been shown to be important in the fermentation of complex plant material (Biddle et al., 2013; Brulc et al., 2009; Ding et al., 2001; Flint et al., 2008). The subtle differences in microbial composition between subjects at the end of fermentation were anticipated since the starting microbial profile in each of the subjects was unique, exerting independent competitions in different communities (Visscher & Stolz, 2005; Long et al., 2013; Heidelberg et al., 2009).

Despite subtle inter-individual differences, there were several taxa that were characterized in all microbiomes. These taxa included members of the *Bifidobacterium, Bacteroides, Prevotella* 9, *Enterococcus, Agathobacter, Roseburia, Ruminococcus* 1, *Dialister, Mitsuokella,* and *Veillonella* genera. Some microbial species from these genera, which include *Bifidobacterium, Enterococcus,* and *Eubacterium,* were also enriched through wheat bran fermentation as shown in previous study (De Paepe et al., 2019). *Agathobacter, Roseburia,* and *Ruminococcus* 1 genera belong to either *Lachnospiraceae* or *Ruminococcaceae* families, potential carbohydrates degraders (Biddle et al., 2013; Flint et al., 1993). Members from genera *Bifidobacterium, Bacteroides,* and *Prevotella* were consistently shown in previous studies (Leitch et al., 2017; De Paepe et al., 2017; De Paepe et al., 2018; De Paepe et al., 2020) to associate with wheat bran fermentation. It is no surprise that *Bifidobacterium, Bacteroides,* and *Prevotella* were associated with WB since studies have recognized that they have plant fiber degrading enzymes or carbohydrate-active enzymes. Studies found carbohydrates hydrolases such as alpha-glucosidases, oligo-1,6-glucosidase, alpha-L-arabinofuranosidase and beta-xylosidase in some of the species from *Bifidobacterium,* suggesting their capability to degrade complex plant-derived oligosaccharides (Barrangou et al., 2009; Pokusaeva et al., 2009; Pokusaeva, Fitzgerald, & van Sinderen, 2011). *Bacteroides* and *Prevotella* are known to encode

multiple CAZymes for cleavage and the utilization of oligosaccharide substrates (Dodd, Mackie, & Cann, 2011). *Prevotella* is also associated with rich dietary fiber and complex carbohydrate diets (De Filippo, et al., 2010; Wu et al., 2011). High dietary fiber intake was also reported to be associated with *Veillonella* (Tomsett et al., 2020). One study also found that 13.6% of core genes in *Enterococcus* are involved in two microbial carbohydrate metabolism pathways (Zhong et al., 2017).

The relative abundance of *Bacteroides, Roseburia, Ruminococcus* 1, and *Dialister* decreased significantly after the initial 24 h fermentation, but recovered and persisted during prolonged fermentation. Perhaps these genera were only competitive when the microbial community was small or there was less competition among the microbes. A similar situation had happened in previous study (Yao, Chen, & Lindemann, 2020); the explanation was that the availability of nutrients, including amino acids and vitamins, in the initial collection of gut micronutrients may impact the activity and abundances of microbiota during the first 24 h of *in vitro* fermentation (Konopka, Lindemann, & Fredrickson, 2015), but their impact would diminish over time.

Gut microbial fermentation of carbohydrates produces short-chain fatty acids (SCFA), which include acetate, butyrate, and propionate (Yao, Chen, & Lindemann, 2020; Tazoe et al., 2008; De Paepe et al., 2019; Haenen et al., 2013). All microbiomes increased SCFA-producing capacity during fermentation of WB but were particularly propionigenic. The propionate production was associated with the higher abundance of propionigenic microbes from *Prevotella, Veillonella* and *Bacteroides* in fecal microbiomes (Poeker et al., 2018; Walker et al., 2005). Microbiome 3 and 4 had higher butyrate production in the control group compared to WB group, this phenomenon could be due to the low pH value of fermented consortia in WB group, ranging

from pH 6.1 to 6.8. Studies from Walker et al. (2005) and Belenguer et al. (2007) suggested that a higher pH environment, around pH 6.5, favored propionate production; while lower pH around 5.5 favored butyrate production.

Unsurprisingly, the total branched chain fatty acid production in the control group was higher compared with the WB group. BCFA (Tazoe et al., 2008) which include isobutyrate and isovalerate, are known to be byproducts of protein fermentation in the gut (Macfarlane & Macfarlane, 2003; Neis, Dejong, & Rensen, 2015). The lower protein fermentation in the WB group could be due to the competition of carbohydrate fermentation and low pH from this study as Macfarlane et al. (1988) suggested a higher protein fermentation has been associated with high pH and low carbohydrates in the gut.

In conclusion, the stepwise *in vitro* fecal fermentation was successfully modified, and the microbiomes were able to maintain or improve their carbohydrate fermentation ability. Since the fermentation steps were under high dilution pressure and digested wheat bran was provided as the sole nutrient source in the WB group, all microbiomes converged to a more similar composition with subtle differences. Almost half of the genera with increased relative abundance among the four microbiomes were from either *Lachnospiraceae* or *Ruminococcaceae*, the potential carbohydrates degraders. The several taxa that were characterized in all microbiomes after 96 h of fermentation with WB included *Bifidobacterium, Bacteroides, Prevotella* 9, *Enterococcus, Agathobacter, Roseburia, Ruminococcus* 1, *Dialister, Mitsuokella,* and *Veillonella* genera. These findings will be useful to develop strategies in improving the fermentation of non-digestible carbohydrates.

2.6. References

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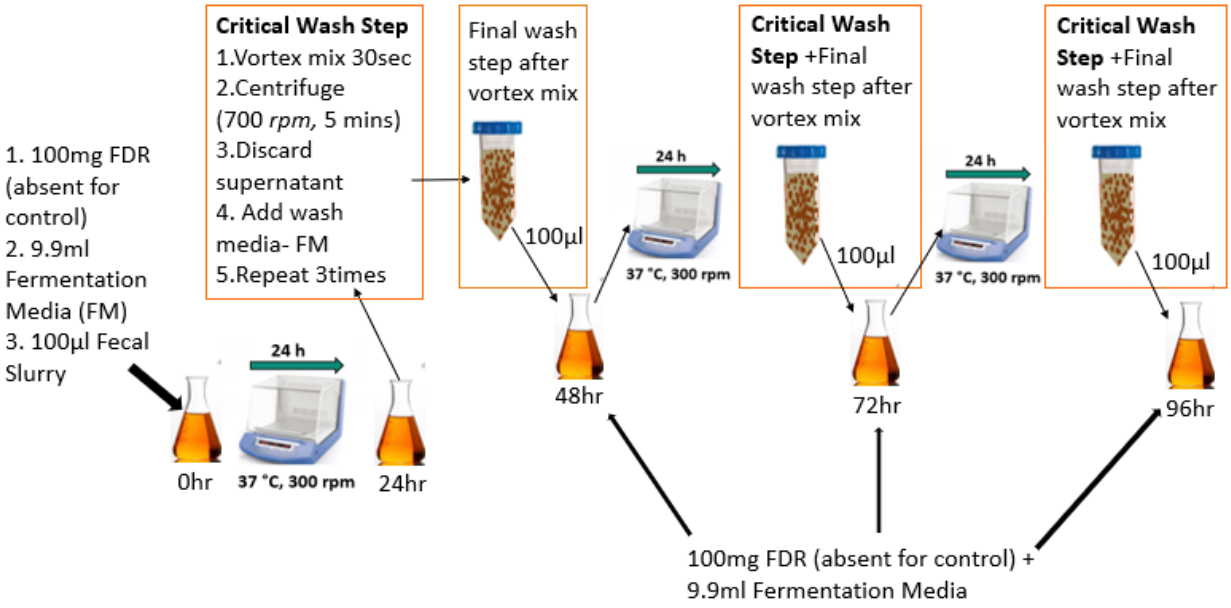

Figure 2.1 Flow chart of modified step wise *in vitro* fermentation. Freeze dried retentate, FDR represented the *in vitro* digested WB.

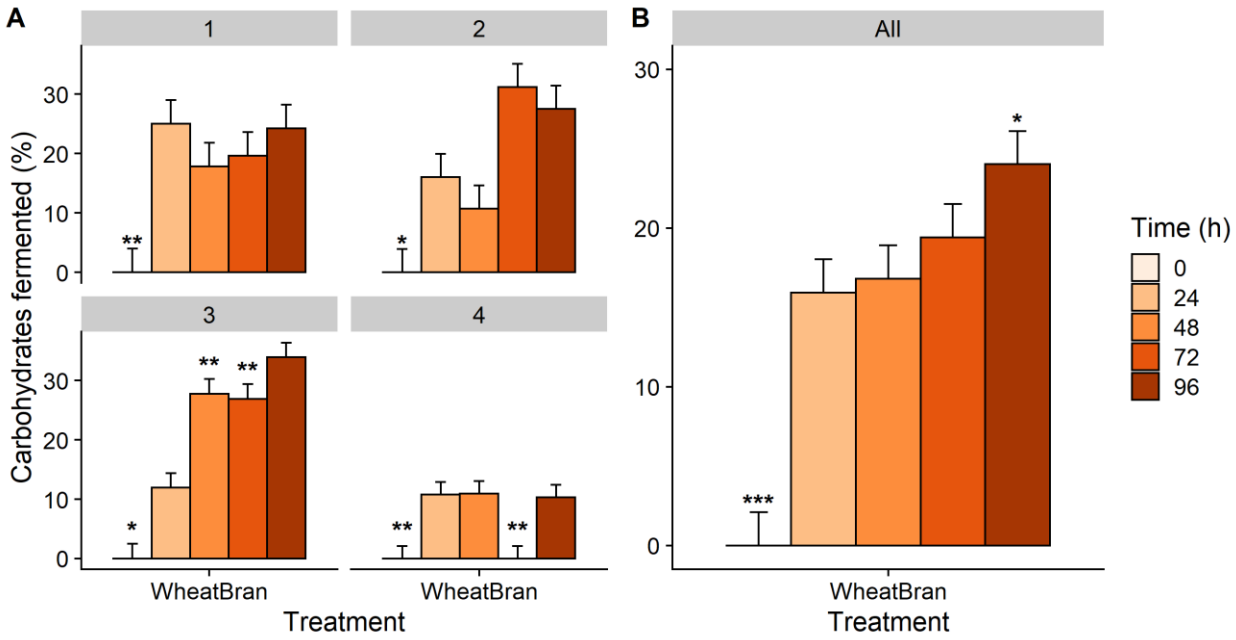

Figure 2.2 The percentage of carbohydrates fermented from WB during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test).

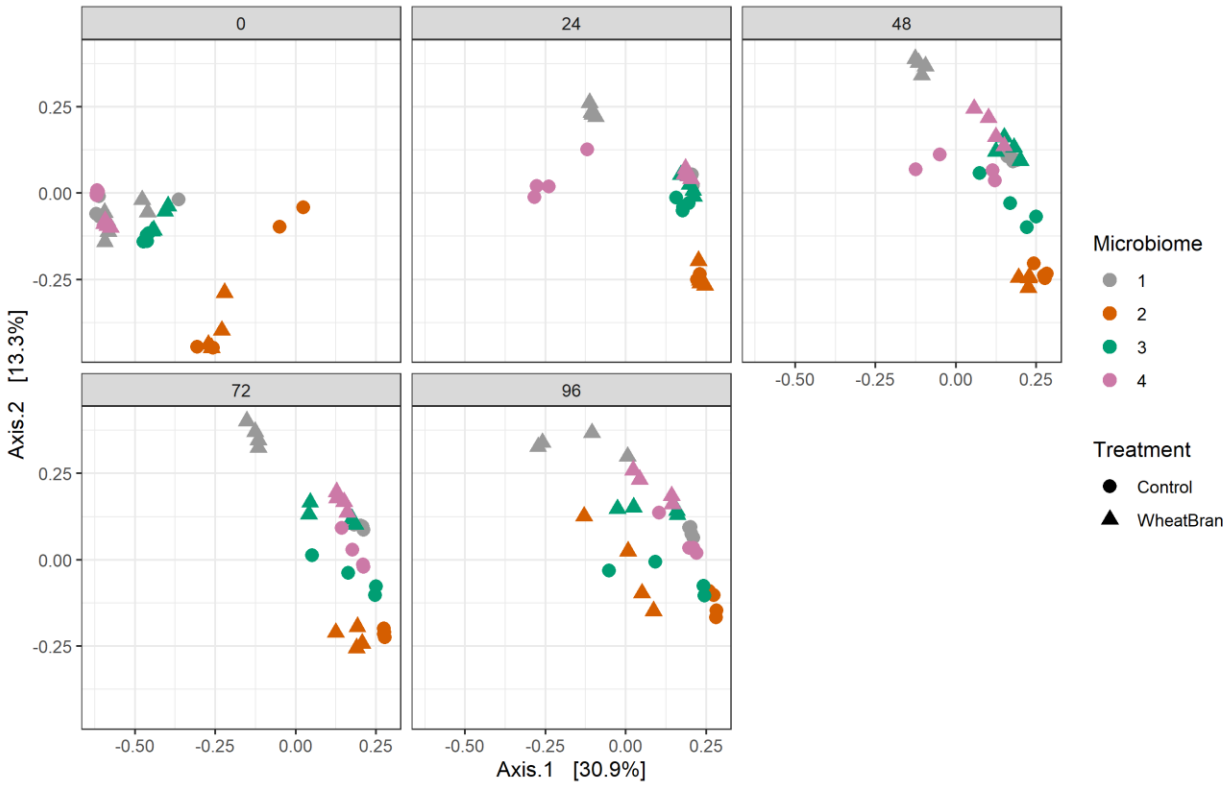

Figure 2.3 β-diversity, principal coordinates analysis (PCoA) biplot calculated based on Bray-Curtis distance among samples grouped by time, 0 h, 24 h, 48 h, 72 h, 96 h.

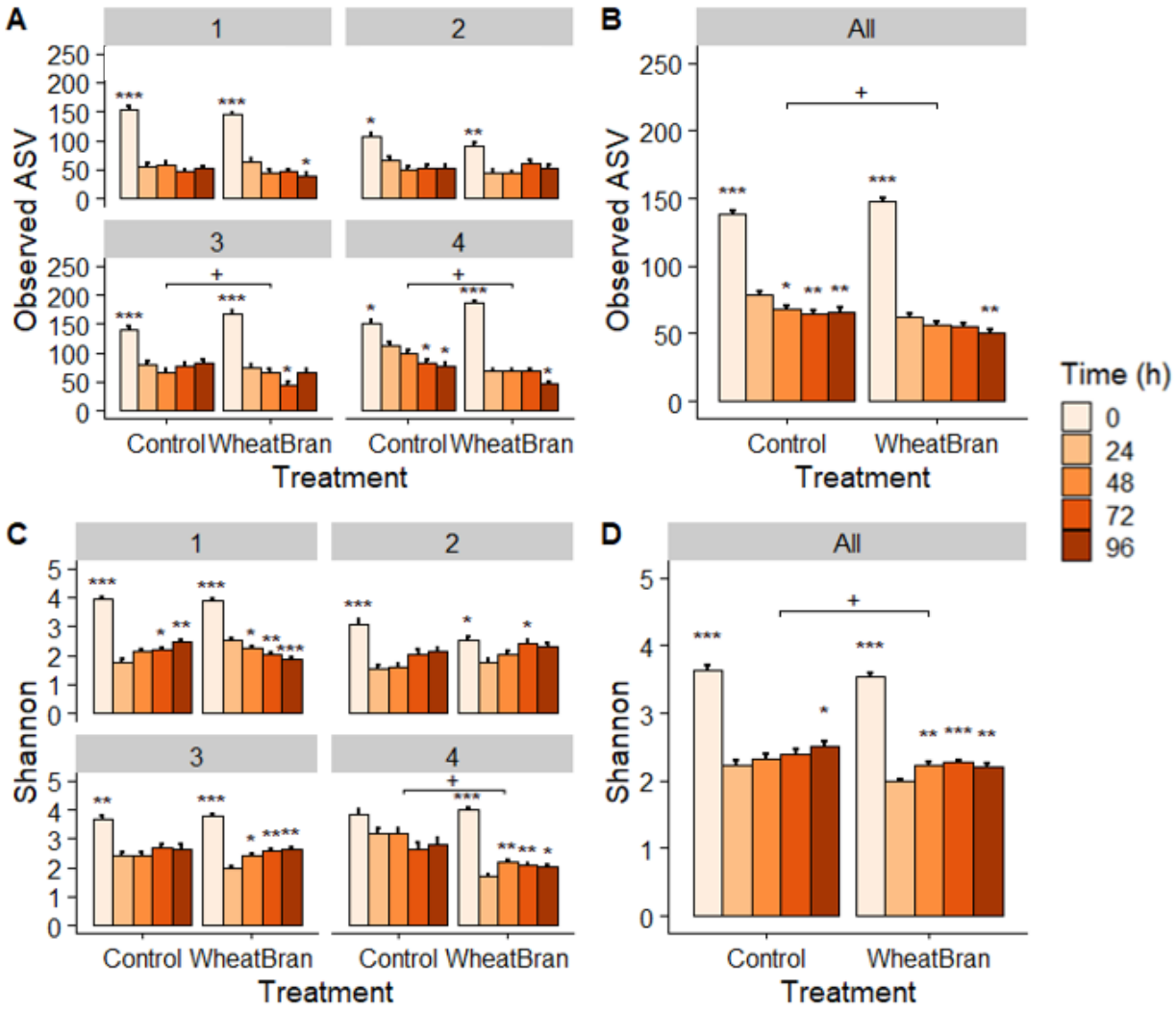

Figure 2.4 α-Diversity- Observed Amplicon Sequence Variances (ASVs) index during each 24 hour period by microbiome (A) and across all microbiomes (B). Shannon-index during each 24 hour period by microbiome (C) and across all microbiomes (D). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.01 for the comparison between treatments, Control and Wheat Bran (Dunnett's test).

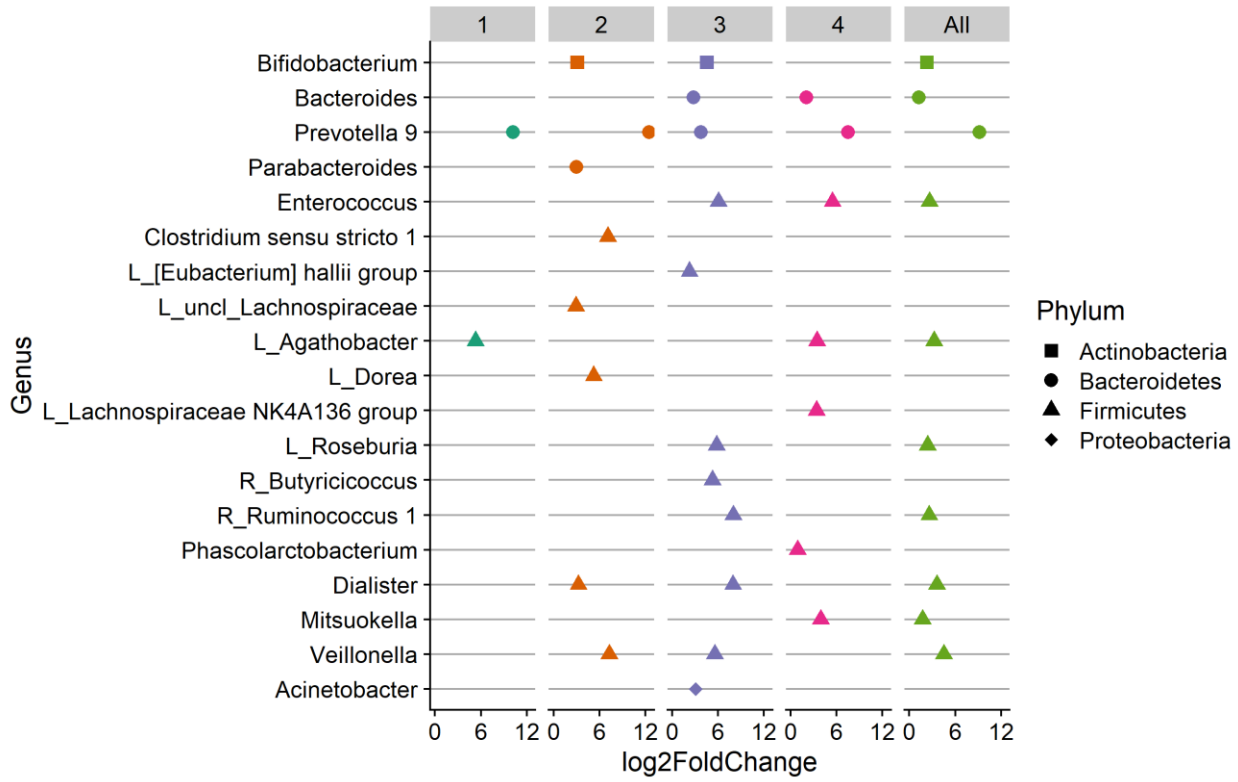

Figure 2.5 Genera with higher abundance in wheat bran treated samples compared with control after 96 h of fermentation. Subpanels represent the results from each of the four microbiomes and from all microbiomes together. The L or R prefix before some genera indicates the family of which that genus belongs, *Lachnospiraceae* or *Ruminococcaceae*, respectively.

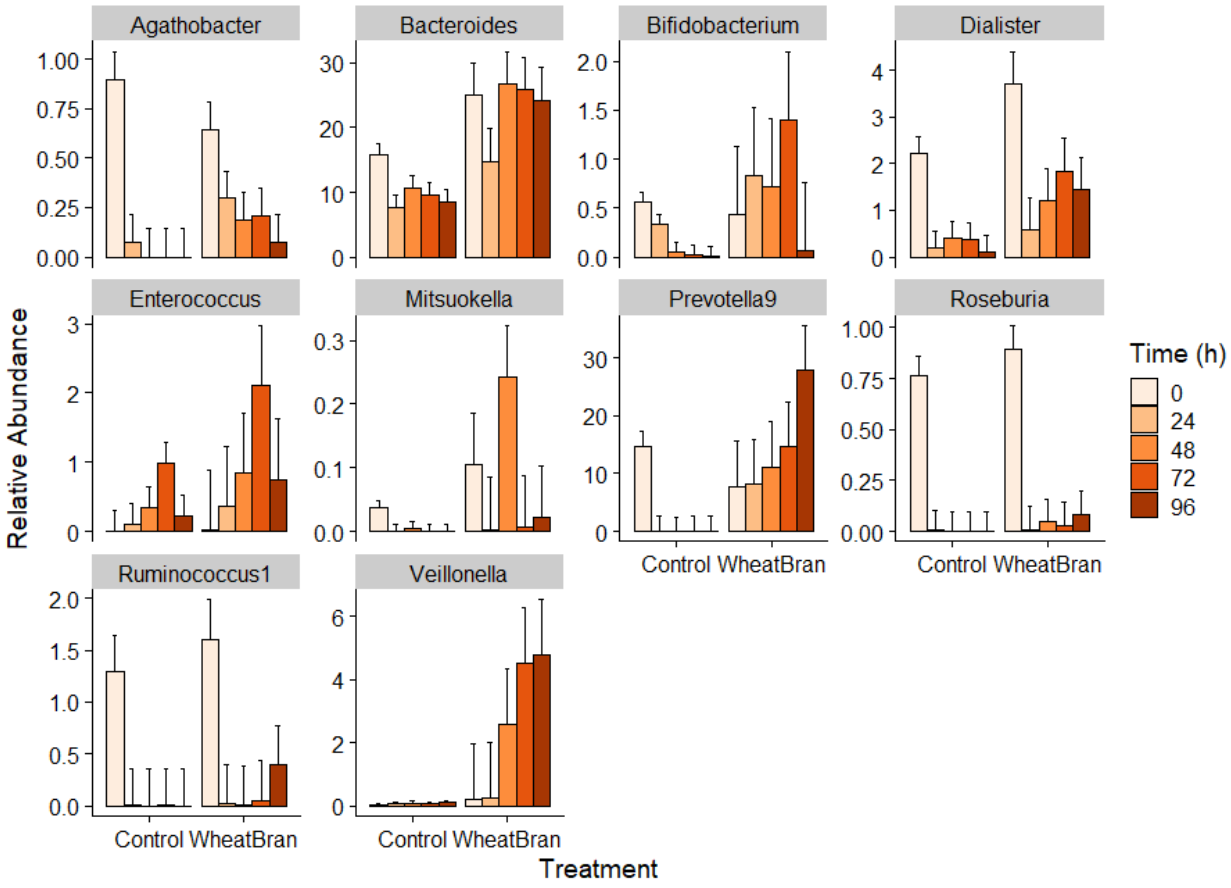

Figure 2.6 The relative abundance of genera *Agathobacter*, *Bacteroides*, *Bifidobacterium*, *Dialister*, *Enterococcus*, *Mitsuokella*, *Prevotella* 9, *Roseburia*, *Ruminococcus* 1, and *Veillonella* across all microbiomes during each 24 h.

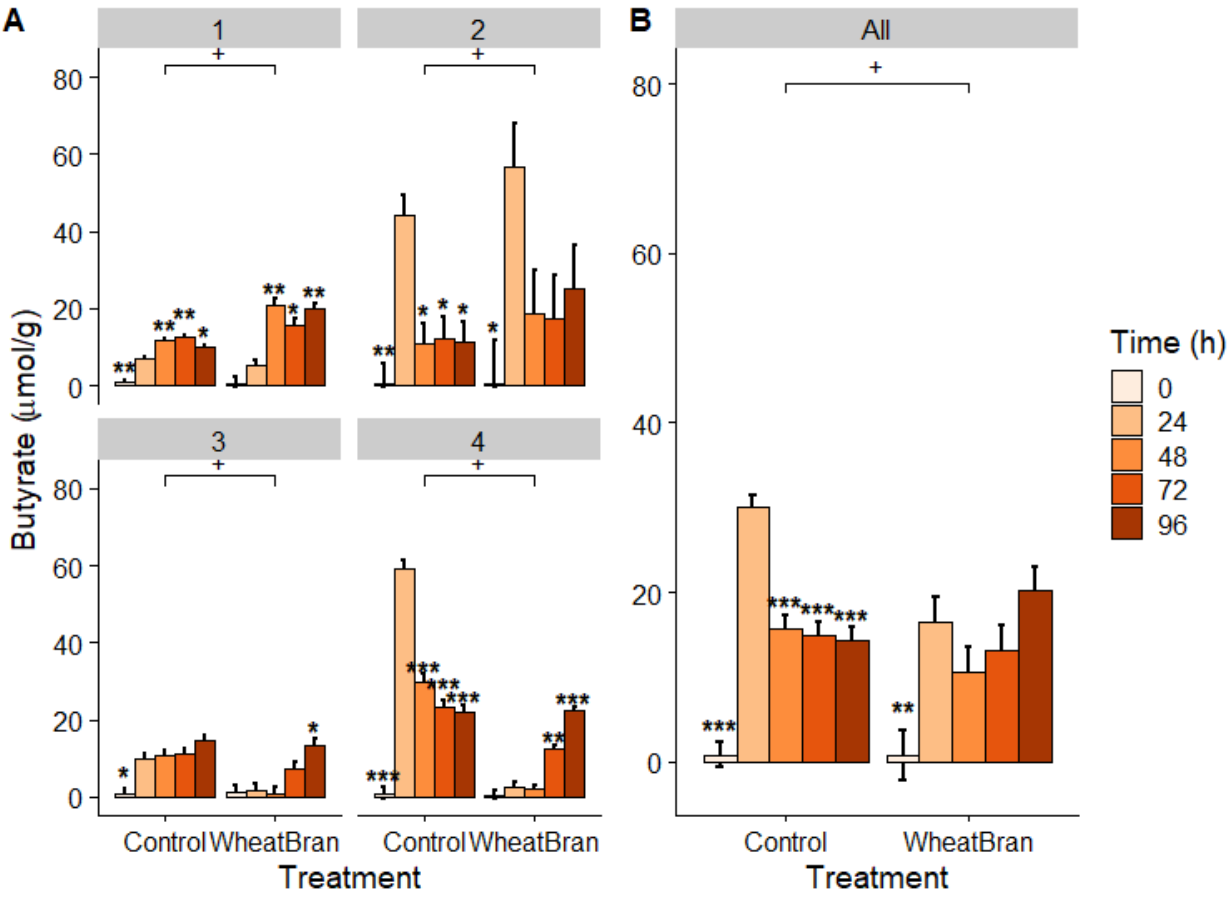

Figure 2.7 Butyrate production during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.001 for the comparison between treatments, Control and Wheat Bran (Dunnett's test).

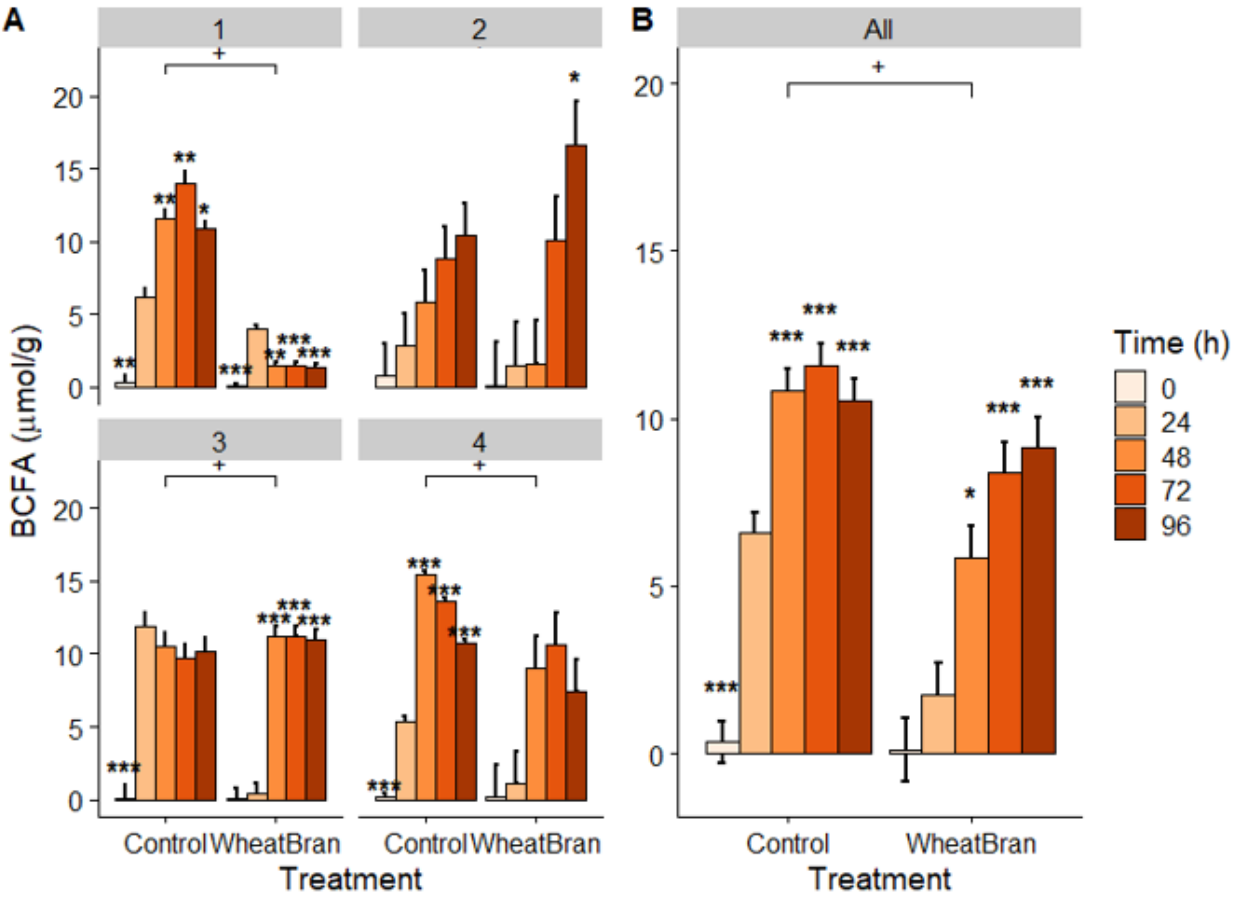

Figure 2.8 Total branched chain fatty acid (isobutyrate and isovalerate) production during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.001 for the comparison between treatments, Control and Wheat Bran (Dunnett's test).

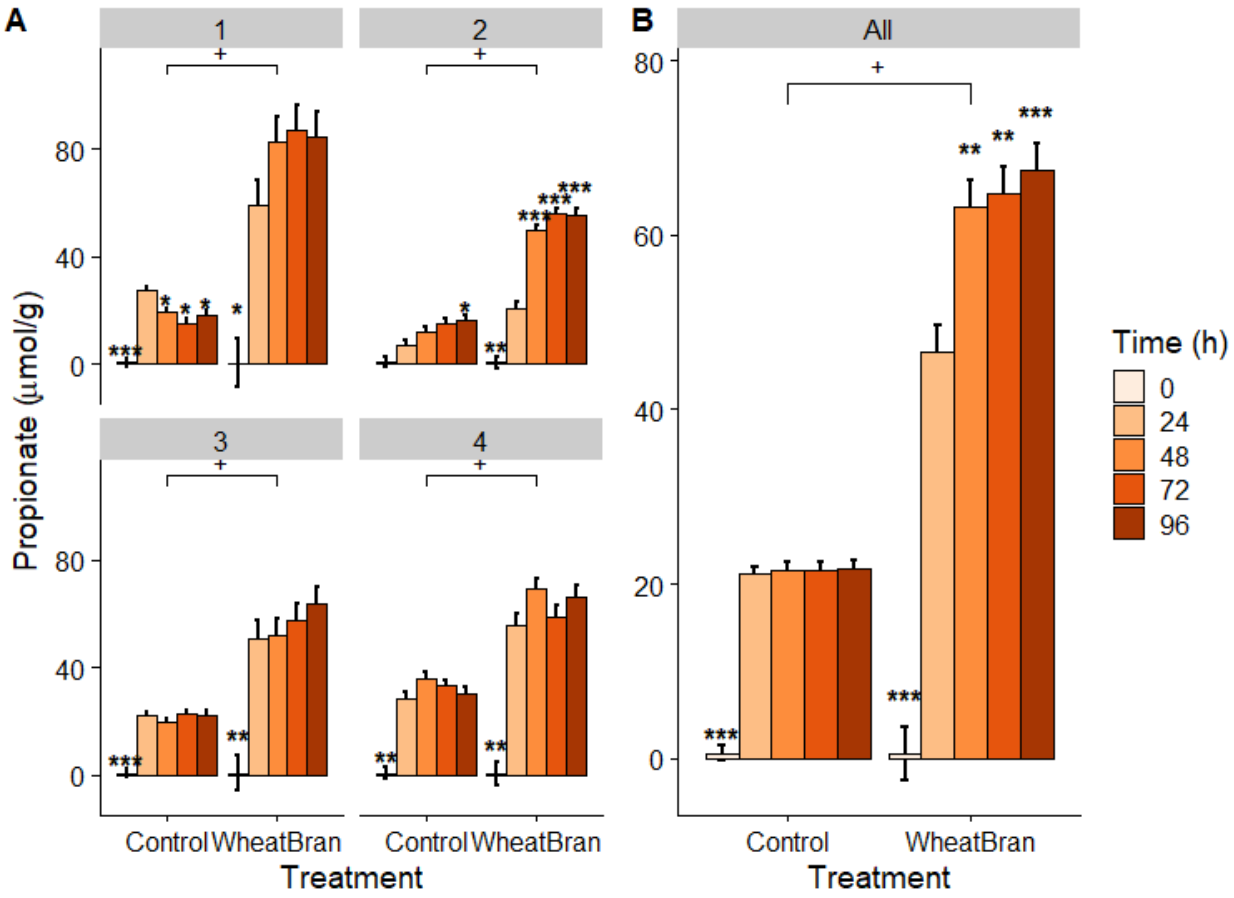

Figure 2.9 Propionate production during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.001 for the comparison between treatments, Control and Wheat Bran (Dunnett's test).

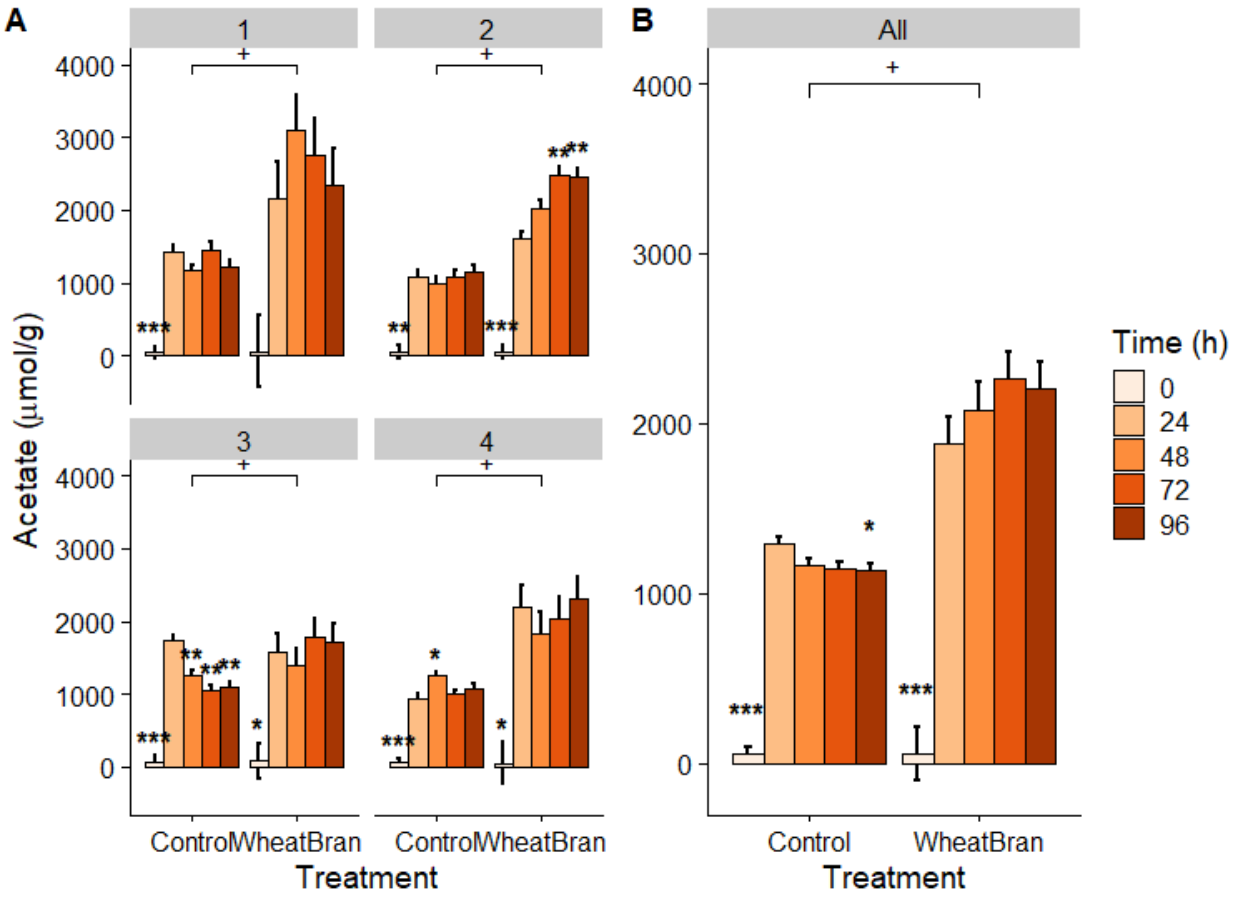

Figure 2.10 Acetate production during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.0001 for the comparison between treatments, Control and Wheat Bran (Dunnett's test).

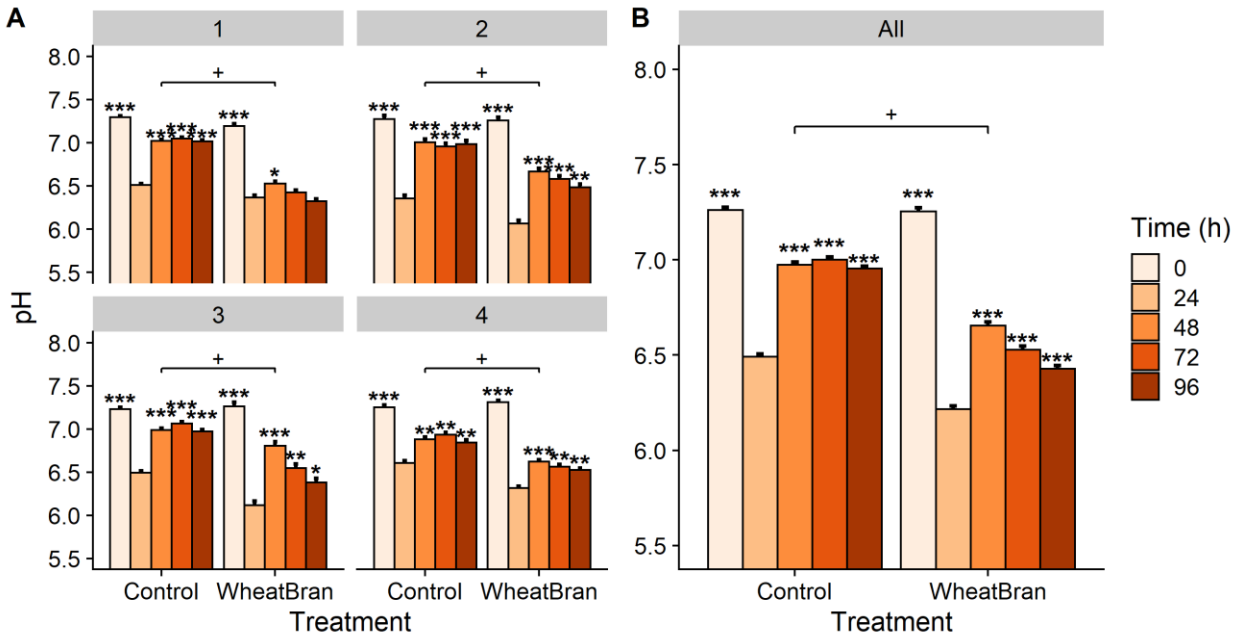

Figure 2.11 The pH value of consortia in the wheat bran group and the control group during each 24 hour period by microbiome (A) and across all microbiomes (B). *shows significant difference at each 24h period from the first 24h period. *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.05 for the comparison between treatment (control group and wheat wheat bran group) with Dunnett's test.

CHAPTER 3 CHARACTERISTICS OF PINTO BEAN-ASSOCIATED MICROBIOTA

3.1. Abstract

The non-digestible carbohydrates (NDC) in PB are incompletely fermented by the gut microbiota. Since diet is a major driver of the gut microbial diversity, a group of core gut microbes were proposed to be associated with the utilization of PB-NDC. To shed some light on this area, my goal for this study was to identify the characteristics and functionalities of PB-associated microbiota using a stepwise *in vitro* fecal fermentation approach. During the stepwise *in vitro* fecal fermentation, PB-associated microbiotas were transferred to new medium containing digested pinto bean at every 24 h for 3 cycles, aimed to select for PB-associated microbes from among four human fecal microbiotas. There was a slow but steady decrease in observed amplicon sequence variants (ASVs) but no significance changes in Shannon diversity index across all microbiomes during PB fermentation. The microbiomes maintained their distinctiveness in microbial composition after 96 h of fermentation with PB, as shown in β-diversity and relative abundance results. At least one of the four microbiomes had elevated relative abundance of *Parabacteroides, Clostridium sensu stricto* 1*, [Eubacterium] fissicatena* group*, Dorea, Hungatella, Lachnospiraceae* ucg-008*, Butyricicoccus,* and *Subdoligranulum* compared with the control. Despite inter-individual differences, there were several taxa that were elevated in all microbiomes compared with the control. These taxa included members of *Prevotella* 9*, Enterococcus, [Ruminococcus] gnavus* group*, [Ruminococcus] torques* group*, Agathobacter, Lachnospiraceae* NK4A136 group*, Roseburia, Dialister, Veillonella,* and uncl_*Enterobacteriaceae*. This indicates that the phenotypic plasticity of the gut microbiome is

often a result of community effects. Carbohydrate utilization of PB-associated microbiota was driven by inter-individual variability over 96 h of fermentation period, with carbohydrates fermented ranging 2.8% to 45.8% ($p<0.05$). All PB-associated communities were SCFA producers and specifically propionigenic. At 96 h, propionate produced by the microbiomes in PB group ranged from 31.2 μmol/g to 59.3 μmol/g ($p<0.05$). PB-associated microbiomes were low producers of branched chain fatty acids; BCFA production was significantly lower in the PB group compared the control group ($p<0.001$). These findings will be useful in developing dietary strategies to modulate the human gut microbiome.

3.2. Introduction

Western diseases including inflammatory bowel disease, type 2 diabetes, cardiovascular diseases, and obesity have been linked to gut health (Remely et al., 2014; Ley, Turnbaugh, Klein, & Gordon, 2006; Scher et al., 2015). The diseases were also found to be associated with diet, the driver of gut microbial composition. Studies suggest that the source of nutrients to nourish the gut microbiota, specifically carbohydrates and proteins, are critical in shaping the gut microbiota (Leitch et al., 2007; Ze, Duncan, Louis, & Flint, 2012; Louis, Hold, & Flint, 2014; Machiels et al., 2014).

Dry beans are an inexpensive source of carbohydrates and proteins that could be utilized by gut bacteria and contribute to gut health and reduce disease risks. In general, dry beans are 20–25% proteins and 50–60% carbohydrates (Aykroyd & Walker, 1982), and the dry basis of cooked pinto beans are approximately 16.8% proteins, 36.5% polysaccharides, 31.1% insoluble fiber, and 28.2 % soluble fiber (Campos-Vega et al., 2009). In addition, dry beans are also rich in essential vitamins, minerals, antioxidants, and antimicrobial phenolic compounds, and low in fat

(Ganesan & Xu, 2017; Kalogeropoulos et al., 2010; Lyimo, Mugula, & Elias, 1992). Previous studies that used dry beans as treatment in both *in vivo* (Winham, Hutchins, & Johnston, 2007; Yao, Cheng, & Ren, 2014) and *in vitro* fermentation (Durak, Baraniak, Jakubczyk, & Świeca, 2013; Mojica & González de Mejía, 2015) linked dry beans to decreased risks of chronic diseases.

Studies suggest that the bioactive peptides from dry beans may be microbiota-active and play an important role in antioxidant and antimicrobial activities. Specifically, studies show that the *in vitro* enzymatic hydrolysis of dry beans and the simulation of gastrointestinal digestion generated bioactive peptides against dipeptidyl peptidase-IV enzyme which aimed in handling type 2 diabetes (Mojica and González de Mejía 2015; Rocha et al. 2014; Rocha et al. 2015). Additionally, bioactive peptides obtained from dry beans by enzymatic protein hydrolysis performed by Ariza-Ortega et al. (2014), presented antioxidant and antimicrobial activity by inhibiting the growth of pathogenic microorganisms such as *Shigella dysenteriae*. Given that dry beans are good source of a variety of nutrients, especially carbohydrates and proteins, the common sources for bacterial fermentation, previous studies that utilized pinto beans as a substrate during *in vitro* fermentation resulted in low pH value and high byproducts of bacterial fermentation, short chain fatty acids (acetate, butyrate and propionate) that are known to promote gut health (Campos-Vega et al., 2009; Chen et al., 2020; Guan et al., 2020).

Pinto bean production has been the highest among other dry beans (black beans, red kidney beans, great northern beans, lima beans, navy beans, pink beans, and small red beans) in recent years, according to USDA National Agricultural Statistics Service (2021). In the year 2020, almost half of the total dry bean production in the US, approximately 0.7 metric tons, was contributed by PB production alone. This massive production of pinto beans provided an

opportunity to introduce them as a potential functional food to modulate gut health. However, despite the United States being the 4th highest bean producer in the world, most Americans do not meet the recommended intake range for beans, peas, and lentils according to the Dietary Guidelines for Americans (2020-2025) (U.S. Department of Agriculture, U.S. Department of Health and Human Services, 2020). In order to better understand the function of dry beans in relation to gut health which eventually reduces the risk of diseases, more research must be carried out on the relationship between dry beans and the gut microbiota.

Considering that different gut microbes encode specialized enzymes to ferment specific nutrient sources (Cantarel, Lombard, & Henrissat, 2012), a group of key microbes was proposed to drive the fermentation of pinto beans. Thus, the goal of this study was to identify the characteristics and functionalities of PB-associated microbiota to maximize the disease-preventing properties of this under-consumed commodity.

3.3. Materials and Methods

3.3.1. Pinto Bean Processing

Dry pinto beans were obtained from a local market and finely milled using a cyclone mill (UDY 3010-030 Cyclone Sample Mill, UDY Corporation Ft. Collins, Colorado) equipped with a 1 mm diameter screen and stored at -80°C until digestion.

3.3.2. Fecal sample processing for *in vitro* fermentation

All procedures involving human subjects were approved by the UNL Institutional Review Board (20160816311EP). Four volunteers donated their fecal samples after signing the consent forms indicating that they had no known gastrointestinal disease, were 19 years of age or older, had not consumed antibiotics or probiotic supplements in the last 6 months, and were not a

regular consumer of yogurt. Fresh fecal samples were weighed and stomach mixed with phosphate-buffered saline containing glycerol as a cryoprotectant (8 g/L sodium chloride, 0.2 g/L potassium chloride, 1.44 g/L disodium phosphate, 0.24 g/L monopotassium phosphate, 100 mL/L glycerol, pH 7.3) in ratio 1:9 (w/v), filtered with filtra bags (Filtra-Bag, Thomas Scientific, New Jersey), and divided into 15 mL aliquots inside of an anaerobic chamber (Bactron X, Sheldon manufacturing, Cornelius, OR, USA) containing 5% H_2, 5% CO_2, and 90% N_2. Aliquots were stored at -80°C until fermentation.

3.3.3. *In vitro* digestion

In vitro digestion was performed as described in Yang, Keshavarzian, and Rose (2013) with minor modifications. Pinto beans (25 g) were boiled in 300 mL of distilled water for 20 min with stirring. Next, 1 M HCl was added to adjust the pH to 2.5, followed by the addition of 10 mL of 1.7% (w/v) pepsin (P7000, Sigma, St Louis, MO, USA) in 50 mM HCl before incubation with orbital shaking at 150 rpm for 30 min at 37°C. Then, 50 mL of 0.1 M sodium maleate buffer (pH 6) containing 1 mM calcium chloride was added and the pH was adjusted to 6.9 with 1 M sodium bicarbonate. Fifty mL of 12.5% (w/v) pancreatin (P7545, Sigma) in sodium maleate buffer was then added followed by 2 mL of amyloglucosidase (3260 U/mL, Megazyme, Bray, Ireland) before incubation with 150 rpm orbital shaking for 6 h at 37 °C. Following digestion, the mixture was transferred to dialysis tubing (molecular weight cutoff, 3500Da) and dialyzed against distilled water at 4 °C for 4 d with a water change at least every 12 h. Then, the dialysis retentate containing the undigested material was freeze dried (FreeZone Tray Dryer, Labconco, Kansas City, MO, USA).

3.3.4. *In vitro* fermentation

Stepwise *in vitro* fecal fermentation was performed according to Kok et al. (2019) with modifications to select for PB-associated microbes (Figure 3.1). First, in a 15 mL centrifuge tube, 100 mg of the freeze dried retentate (*in vitro* digested PB) was suspended in 9.9 mL of sterile fermentation medium for 30 min. Tubes were prepared in duplicate per replication (four tubes total); one tube was used for carbohydrate quantification and the other tube was used for microbiota composition, pH measurement, and SCFA analysis. A control was also prepared that did not contain PB. Next, 0.1 mL of each of four fecal slurries processed earlier were added separately to the PB slurry or control and incubated at 37 °C with orbital shaking (300 rpm) for 24 h. The fermentation medium used in the control group contained (per L): peptone (2 g, Fisher Scientific, Waltham, MA), yeast extract (2 g, Fisher Scientific, Waltham, MA), bile salts (0.5 g, Oxoid, Cheshire, England), sodium bicarbonate (2 g), sodium chloride (0.1 g), dipotassium phosphate (0.08 g), magnesium sulfate heptahydrate (0.01 g), calcium chloride hexahydrate (0.01 g), L-cysteine hydrochloride (0.5 g, Fisher Scientific, Waltham, MA), hemin (50 mg dissolved in DMSO), Tween 80 (2 mL, Fisher Scientific, Waltham, MA), vitamin K (10 μL, dissolved in ethanol, Alfa Aesar, Haverhill, MA), and 0.025% (w/v) resazurin solution (4 mL, dissolved in water, Alfa Aesar, Haverhill, MA), prepared according to Yang, Keshavarzian, and Rose (2013). A liter of fermentation medium prepared with 2 g of peptone would contain approximately 0.0198 g of nitrogen in 9.9 mL of fermentation media. Since in each centrifuge tube of the PB group, the 100 mg of freeze dried retentate (*in vitro* digested PB) already contained approximately 0.057 g of nitrogen, no peptone was added to the recipe of fermentation medium used for the PB group.

At 24 h, the duplicate tubes underwent a “critical wash step” to select for a PB-associated consortia, according to De Paepe *et al.* (2017) and De Paepe *et al.* (2019) with modification.

First, tubes were centrifuged at 700 rpm, 5 min to pellet the PB and associated microbes, the supernatant was separated for pH measurement and SCFA analysis. Next, the pellets were washed three times with 5 mL of fresh fermentation medium using the same centrifugation conditions. After discarding about 3 mL of the supernatant (wash medium) from the last centrifugation (leaving PB-associated microbes suspended in about 2 mL of fermentation medium), one of the duplicate tubes were removed from the anaerobic chamber and immediately frozen in liquid nitrogen for future carbohydrates analysis ; from the other tube 0.1 mL of PB-associated consortia suspension was transferred in duplicate into fresh centrifuge tubes containing 100 mg of freeze dried retentate and 9.9 mL of fermentation media. This mixture was fermented for another 24 h. At the end of this fermentation period, the tubes were designated as 48 h tubes and the same freezing and critical washing steps were performed as described for the 24 h tubes. This was continued for a total of 96 h of fermentation. The entire process was conducted inside an anaerobic hood, including centrifugation, washing, inoculation, and fermentation. Frozen tubes were then stored at -80°C for fermentation analyses use.

3.3.5. Fermented carbohydrates

The residues of carbohydrates that were not fermented by the microbiomes were hydrolyzed according to Englyst, Quigley, Hudson, and Cummings (1992). Tubes designated for carbohydrate analysis were centrifuged at 14,000 rpm for 5 min and the pellet was separated from supernatant and both were retained. Then, approximately 4 mL of concentrated sulfuric acid was added to the pellet to obtain a final concentration of 12 M. After vortex mixing, the mixture was incubated at 35°C for 1 h with occasional mixing. The reserved supernatant was added back to the hydrolyzed pellet and 25 mL distilled water was used to quantitatively rinse any remaining supernatant back into the tube with the hydrolyzed pellet. This resulted in a final

sulfuric acid concentration of 2 M. The mixture was then placed in a boiling water bath for 1 h. The concentration of carbohydrates in the hydrolyzed samples were then diluted in ratio 1:10 and measured by the phenol-sulfuric acid method using diluted glucose in the concentration of 0.01, 0.05, 0.1, 0.15, and 0.21 mg/mL as the standards (Dubois et al., 1956). The percentage of carbohydrates fermented was calculated based on the difference between the carbohydrates supplied at the beginning of each 24 h period and the carbohydrates remaining after fermentation divided by the quantity at the beginning of fermentation. Carbohydrate utilization in the first 24 h of fermentation was compared with that of other time points to determine whether the microbial community changed in its ability to ferment NDC from PB.

3.3.6. Microbiota composition

DNA extraction was done with BioSprint 96 One-For-All Vet Kit (Qiagen) according to the BioSprint 96 One-For-All Vet Handbook. Prior to DNA extraction, the fermented samples were thawed on ice for around 30 mins, followed by centrifugation at 14,000 rpm, 5 mins to obtain the pellets of microbes. The pellets were suspended in warmed ASL stool lysis buffer and transferred into 96 deep well bead-beating plates. Plates were then bead beaten 4 times (4 mins, 1800 rpm) with rest intervals of 10 mins, followed by 70 °C incubation in water bath for 10 mins. While bead beating, wash plates were prepared according to the manual. Then, 100 µL of samples were added into an S-block loaded with 40 µL proteinase K, incubated in water bath at 70°C for 10 mins. Finally, for 96 samples, RLT mixture containing buffer RLT (35 mL), isopropanol (35 mL). Mag Attract Suspension G (3 mL), and buffer AVE with carrier RNA (310 µL) was added into the warmed S-block (600 µL of RLT mixture in each well) before placing into BioSprint 96 for automated extraction. Extracted DNA samples were then subject to amplicon sequencing of the V4 region of the 16S rRNA gene on the Illumina MiSeq platform

using MiSeq Reagent kit v2 (2 X 250 bp) following the procedure in Kozich et al. (2013). Illumina data were processed according to the following steps. Utilizing the QIIME 2 platform, the sequence analysis was performed once sequences were demultiplexed and barcodes were removed (Bolven et al., 2019). Next, DADA2 was used to perform sequence quality control, trimming, chimera removal and denoising (Callahan et al., 2016). To maintain sequence qualities above a phred score of 30, forward and reverse reads were truncated to 245 and 160 bp. Sequences were dereplicated into 100% ASVs with DADA2 for exact sequence matching. SILVA database46 were used to assign the taxonomy (Quast, C. et al., 2013). Before statistical and diversity analyses, the reads were rarefied to a sampling depth of 9,000. QIIME2 was used to calculate the diversity metrics of the fecal and fermented samples.

3.3.7. Short-chain fatty acids

SCFAs were extracted from the supernatant of fermented samples separated after the centrifugation performed prior to DNA extraction for 16S sequencing and measured by gas chromatography as in Hartzell, Maldonado-Gómez, Hutkins, & Rose (2013). In short, 0.4 mL of supernatant from fermented samples, 0.1 ml of 7 mM 2-ethylbutyric acid in 2 M potassium hydroxide, 0.2 ml of 9 M sulfuric acid, and sodium chloride were added and vortex mixed. Then, 0.5 mL of diethyl ether was added and centrifuged at 14,000 rpm, 2 min. The separated top layer, diethyl ether phase, was collected and injected into a gas chromatograph (Clarus 580, PerkinElmer, MA USA) with capillary column (Elite-FFAP, 15 m×0.25 mm inner diameter×0.25 μm film thickness, PerkinElmer, Waltham, MA, USA) and the SCFA were detected by a flame ionization detector at 240 °C. SCFA data were then quantified by calculating response factors for each SCFA relative to 2-ethyl butyric acid using injections of pure standards.

3.3.8. pH analysis

Supernatant from fermented consortia at 24 h, 48 h, 72 h, 96 h and supernatant of consortia mixture before fermentation at 0 h were collected for pH measurement. This supernatant was obtained after the first centrifugation step in the "critical wash step" mentioned in the *in vitro* digestion method. Measurement was carried out using a pH probe (FiveEasy F20, Mettler Toledo, Switzerland).

2.3.9. Statistical analysis

For carbohydrate, SCFA, and α-diversity data, a two factor ANOVA was performed where subject and time were the factors. Within each subject and across all subjects, Dunnett's test was used to determine significant differences between samples using the 24 h fermented samples as control, and to determine significant differences between treatment group (pinto bean) and control group (without pinto bean). All ANOVAs were run and analyzed using SAS software (version 9.4, Cary, NC USA).

The community sequencing data was analyzed with DESeq2 package (Love & Huber & Anders, 2014) in RStudio (version 3.4.3). Features that were present at <0.1% in all samples were removed prior to the analysis. The remaining features represented in 139 genera were used for analyses. The model analyzed differences in taxonomic composition between the control group and the pinto bean group at 96 h of fermentation. The model was run across all microbiomes, correcting for microbiome (treatment + microbiome) and also run by microbiome where the data were grouped by microbiome.

3.4. Results

3.4.1. Carbohydrate utilization

The PB-associated microbiomes fermented 2.8% to 45.8% of the total NDC supplied during each 24 h period of fermentation (Figure 3.2). The result was different based on microbiome. Microbiome 1 significantly improved carbohydrate utilization capability over 96 h of stepwise *in vitro* fermentation compared with the first 24 h. Microbiome 2 and 4 maintained their carbohydrate utilization capabilities; while microbiome 3 had diminished carbohydrate utilization capability after 96 h of fermentation compared with the first 24 h.

3.4.2. Microbiota diversity

Principal coordinates analysis of Bray-Curtis distances among samples showed major clustering by time and by microbiome, as expected. Within a microbiome, there was some clustering based on treatment (Figure 3.3). In terms of observed ASVs, there was an overall decreasing pattern in observed ASVs in both the control and the PB group, however, the PB group was significantly lower in observed ASVs compared to the control (Figure 3.4). Shannon diversity also decreased during the first 24 h of fermentation; however, in contrast to observed ASVs, most microbiomes increased in Shannon diversity at subsequent time points (Figure 3.4).

3.4.3. Microbiota composition

Abundances of genera in the PB-associated microbiomes were compared with the control microbiomes containing no PB. The analysis at 96 h of fermentation showed significantly higher abundance of several genera depending on starting microbiome (Figure 3.5). With inter-individual differences, the microbiomes had significantly higher relative abundances of

Parabacteroides, Clostridium sensu stricto 1, *[Eubacterium] fissicatena* group, *Dorea, Hungatella, Lachnospiraceae* ucg-008, *Butyricicoccus,* and *Subdoligranulum*. Analysis across all microbiomes showed higher relative abundances of *Bifidobacterium, Bacteroides, Prevotella* 9, *Enterococcus, [Ruminococcus] gnavus* group, *[Ruminococcus] torques* group, *Agathobacter, Lachnospiraceae* NK4A136 group, *Roseburia, Dialister, Veillonella,* and uncl_*Enterobacteriaceae* (Figure 3.5). There was a total of 20 microbial genera associated with PB-associated microbiomes (Figure 3.6). More than half of these genera were from the *Lachnospiraceae* and *Ruminococcaceae*.

3.4.4. Gut metabolites and pH

Gut microbial metabolite production varied among microbiomes (Figures 3.7-3.10). Butyrate produced by each of the microbiomes treated with PB was significantly higher ($p<0.05$) at 96 h when compared to 24 h with an exception of microbiome 2 (Figure 3.7(A)). The butyrate production averaged across all microbiomes in the PB group at 96 h was not significantly higher compared to 24 h, due to the unusually high value at 24 h from microbiome 2 (Figure 3.7(B)). Across all microbiomes, the butyrate production in PB group was significantly higher than control group ($p<0.05$). Branched chain fatty acid (BCFA), the total of isobutyrate and isovalerate production, in the PB group remained low at 48 h, 72 h, 96 h (non-significantly different) when compared to 24 h of fermentation. Overall, the average of BCFA production in control group was higher ($p<0.001$) compared to the treatment group (Figure 3.8 (B)).

Additionally, all microbiomes had significantly increased propionate ($p<0.01$) production over time in the PB group (Figure 3.9). Across all microbiomes, the PB group had significantly higher ($p<0.05$) propionate production (Figure 3.9 (B)) compared to the control group. In the PB

group, while microbiomes 3 and 4 maintained acetate production at 96 h when compared to 24 h microbiomes 1 and 2 produced significantly higher ($p<0.01$) acetate at 96 h when compared to 24 h (Figure 3.10). Across all microbiomes, the PB group had significantly higher ($p<0.0001$) acetate production (Figure 3.10 (B)) compared to the control group.

The pH values of all microbiomes in the PB group ranged from 5.7 ± 0.04 to 7.4 ± 0.04 and were significantly lower ($p<0.0001$) than the control group (Figure 3.11). Microbiomes in the PB group shared the same trend. The pH values were the lowest at 24 h, then increased at 48 h, followed by a decreasing trend from 72 h to 96 h.

3.5. Discussion

Literature on *in vitro* fermentation of dry bean (Chen et al., 2020; Guan et al., 2020; Rovalino-Córdova et al., 2020) is limited, compared to *in vivo* feeding trials on other edible beans (Borresen et al., 2017; Fernando et al., 2010; Finley et al., 2007; Ojo et al., 2021; Sheflin et al., 2017; Smith et al., 2006). Additionally, there is only one previous study that has attempted to identify PB-associated microbiomes with human fecal microbiota (Guan et al., 2020). To identify the characteristics of PB associated microbiomes, we implemented a modified step-wise *in vitro* fermentation strategy. The digested PB was fermented with fecal microbiomes from four human subjects. Every 24 h the PB-associated microbiotas were transferred to new medium containing digested pinto bean as sole nutrient under high dilution pressure (1:100). This condition was most likely responsible for the decrease in diversity richness (observed ASVs index) across all four microbiomes in the PB group. The Shannon index across all microbiomes showed a maintained diversity evenness throughout the fermentation period from 48 h to 96 h, after a significant drop at the initial 24 h of fermentation. Additionally, the comparison of Shannon index between PB group and control group showed no significant difference, indicating

no differences in terms of both species diversity and uniformity between the treatment groups. A similar finding was also observed in another study conducted with *in vitro* fermentation of red kidney beans (Rovalino-Córdova et al., 2020)

The results from β-diversity showed shifts during fermentation with clustering based on microbiomes and treatment (PB group or control group). Similar clustering effect based on treatment was also noticed in other studies conducted with PB substrates (Guan et al., 2020; Ojo et al., 2021)

At the end of 96 h of fermentation, all microbiomes in PB group retained their individuality; each of the four microbiomes was characterized by different lists of gut microbes. At least one of the four microbiomes had elevated members of the *Parabacteroides* (microbiome 2), *Clostridium sensu stricto* 1 (microbiome 2), *[Eubacterium] fissicatena* group (microbiome 1), *Dorea* (microbiome 2 & 3), *Hungatella* (microbiome 2 & 3), *Lachnospiraceae* ucg-008 (microbiome 4), *Butyricicoccus* (microbiome 3), and *Subdoligranulum* (microbiome 4). All these gut microbes except *Parabacteroides* and *Clostridium sensu stricto* 1 belong to either *Lachnospiraceae* or *Ruminococcaceae*. Both families have been shown to be important in the fermentation of complex plant material (Biddle et al., 2013; Ding et al; 2001; Flint et al; 2008). Previous studies on pinto beans with both *in vitro* and *in vivo* methods also showed high relative abundance of microbes from *Lachnospiraceae* in the PB group (Guan et al., 2020; Ojo et al., 2021). Since both *Lachnospiraceae* and *Ruminococcaceae* have been linked with dietary fiber fermentation, the enriched microbiomes were, as expected, shaped through the fermentation of PB.

Despite interindividual differences, there were several taxa that were elevated in all microbiomes. These taxa included members of the *Bifidobacterium, Bacteroides, Prevotella* 9,

Enterococcus, [Ruminococcus] gnavus group, *[Ruminococcus] torques* group, *Agathobacter, Lachnospiraceae* NK4A136 group, *Roseburia, Dialister, Veillonella,* and uncl_*Enterobacteriaceae*. *[Ruminococcus] gnavus* group, *[Ruminococcus] torques* group, *Agathobacter, Lachnospiraceae* NK4A136 group, and *Roseburia* were similar to some microbes mentioned previously, belonging to either the *Lachnospiraceae* or *Ruminococcaceae* and potential carbohydrate degraders (Biddle et al., 2013; Flint et al., 1993). By incorporating PB into a mouse diet, Ojo et al. (2021) was able to detect significantly higher relative abundance of members from the *Lachnospiraceae*, which positively correlated with antimicrobial peptide genes (Reg3 γ and Reg3 β) and members from the *Lachnospiraceae* NK4A136 group which positively correlated with Reg3 β. It is no surprised that up-regulated antimicrobial peptide genes were found to be associated with PB fermentation as PB is a good source of phenolic compounds, a natural antimicrobial agent (Câmara, Urrea, & Schlegel, 2013; Jurd, King, Mihara, & Stanley, 1971). Literature has shown that members from *Bifidobacterium, Bacteroides,* and *Prevotella* 9 contain carbohydrate hydrolases or encode multiple CAZymes for cleavage and the utilization of oligosaccharide substrates (Barrangou et al., 2009; Pokusaeva et al., 2009; Pokusaeva, Fitzgerald, & van Sinderen, 2011; Dodd & Mackie & Cann, 2011). One study also found that 13.6% of core genes in *Enterococcus* are involved in two microbial carbohydrate metabolism pathways (Zhong et al., 2017).

The relative abundance of *[Ruminococcus] gnavus* group, *Roseburia, Dialister* and *Lachnospiraceae* NK4A136 group decreased significantly after the initial 24 h fermentation, but recovered and persisted during prolonged fermentation. Perhaps these genera were only competitive when the microbial community was small or there was less competition among the microbes. A similar situation happened in a previous study conducted with wheat bran (Yao,

Chen, & Lindemann, 2020). The explanation was that the availability of nutrients, including amino acids and vitamins, in the initial collection of gut micronutrients may impact the activity and abundances of microbiota during the first 24 h of in vitro fermentation (Konopka, Lindemann, & Fredrickson, 2015), but their impact would diminish over time.

The fermentability of PB carbohydrates was different among the 4 microbiomes during 96 h of fermentation. Campos-Vega et al. (2009) suggested the carbohydrate constituents from PB played a major role in influencing the pH and SCFA production of in vitro fermentation.

All microbiomes increased SCFA-producing capacity during fermentation of PB, but were particularly propionigenic. The propionate production may be associated with the consortia pH value in the PB group at 96 h, in average ranging 6.0 to 6.3 across all microbiomes. This is supported by Walker et al., 2005 and Belenguer et al., 2007, who suggested that a higher pH environment, around pH 6.5, favored propionate production; while lower pH around pH 5.5 favored butyrate production. The propionate production was associated with the higher abundance of propionigenic microbes from *Prevotella, Veillonella* and *Bacteroides* in fecal microbiomes (Poeker et al., 2018; Walker et al., 2005). Studies have found *Bacteroides* to be potentially saccharolytic and produce lactic and succinic acids, which are intermediate products of carbohydrate fermentation that are further metabolized to butyric and propionic acids (Louis, Scott, Duncan, & Flint, 2007; Salonen et al., 2014). Although the propionate production among the PB group was about twice as much as butyrate production, the butyrate production in the PB at 96 h was significantly higher than the initial 24 h. The increased butyrate may be associated with the increased members of *Lachnospiraceae* family in the PB group, which are known to be butyrogenic (Meehan & Beiko, 2014)

Protein and amino acid fermentation in the large intestine has been known to produce branched chain fatty acids (BCFA), which include isobutyrate and isovalerate (Macfarlane & Macfarlane, 2003; Neis, Dejong, & Rensen, 2015). Although PB is a good source of protein, the BCFA production in the PB group was significantly lower than the control group. The lower BCFA production in the PB group was likely due to the abundant carbohydrates that were available for fermentation instead as higher protein fermentation has been associated with high pH and low carbohydrates in the gut (Macfarlane et al., 1988).

In conclusion, the stepwise *in vitro* fecal fermentation strategy was able to enrich for PB associated microbiomes that were mostly carbohydrate fermenters instead of protein fermenters, evident by the significantly higher SCFA production and lower BCFA in the PB group compared to the control group. While all subjects' microbiomes received the same substrate (PB) in each transfer at 48 h, 72 h, and 96h, the microbiomes did not converge to a more similar composition; they maintained their distinctiveness. The several taxa that were characterized across all four microbiomes in the PB group at 96 h of fermentation included members of the *Bifidobacterium, Bacteroides, Prevotella* 9, *Enterococcus, [Ruminococcus] gnavus* group, *[Ruminococcus] torques* group, *Agathobacter, Lachnospiraceae* NK4A136 group, *Roseburia, Dialister, Veillonella,* and uncl_*Enterobacteriaceae*. These findings will be useful in developing dietary strategies to modulate the human gut microbiome.

3.6. References

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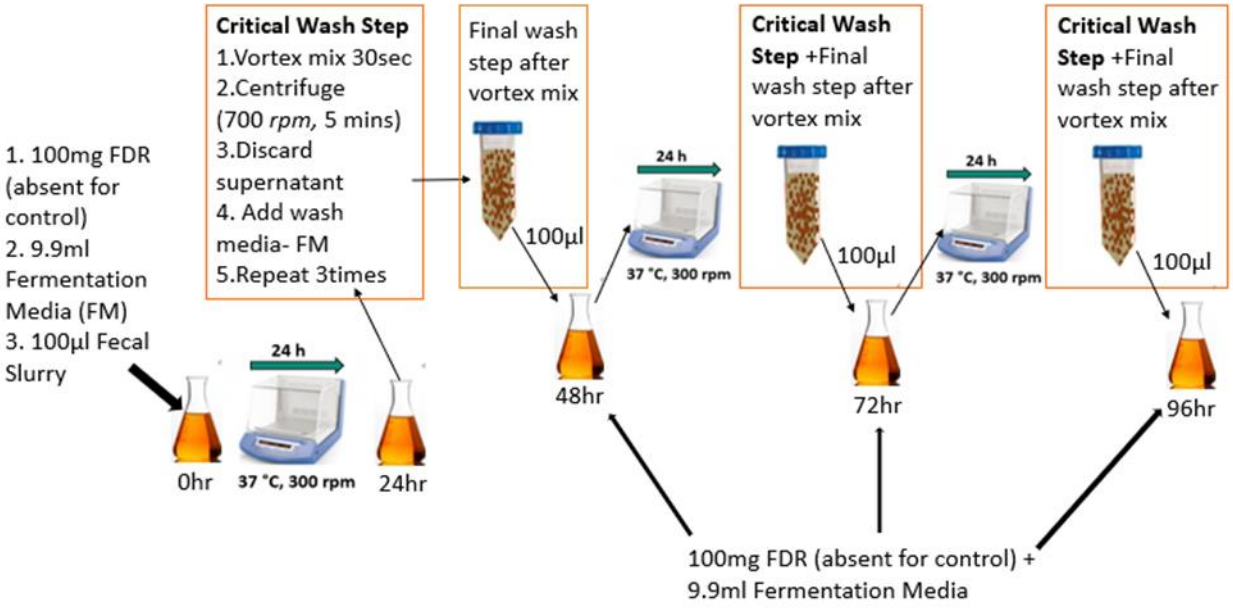

Figure 3. 1 Flow chart of modified step wise *in vitro* fermentation. Freeze dried retentate (FDR) represented the *in vitro* digested PB.

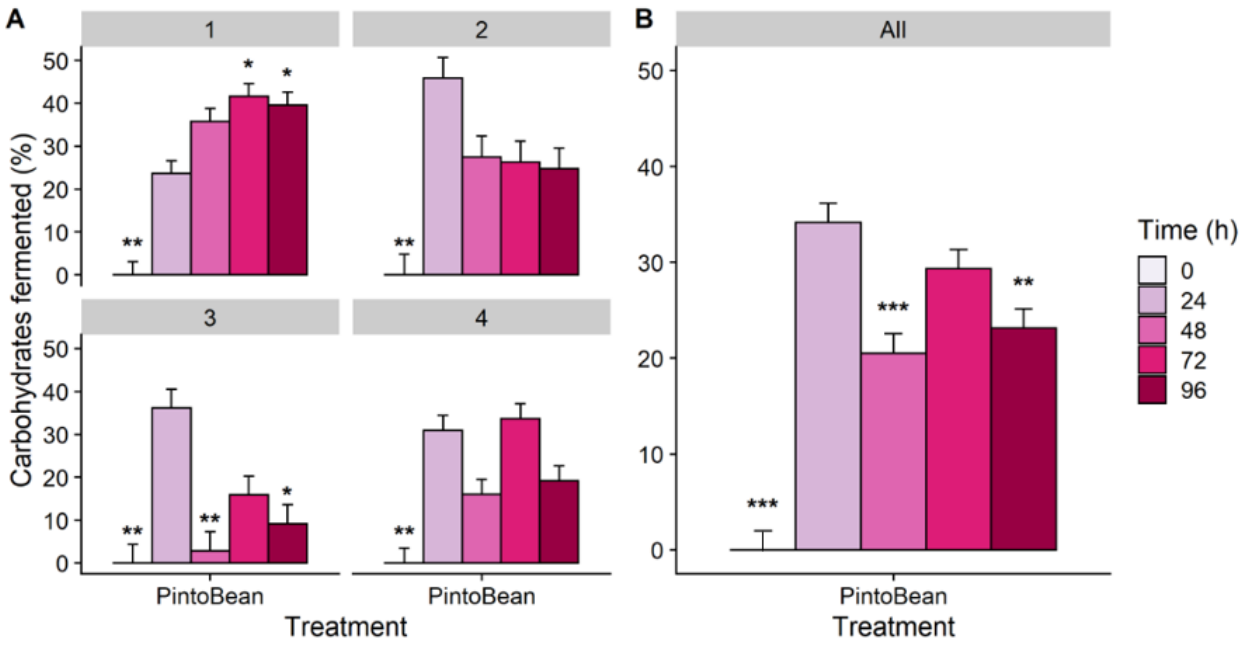

Figure 3.2 The percentage of carbohydrates fermented by pinto bean group across 4 cycles of 24 h fermentation periods. Each of the four smaller barplots on the left represent each of four subjects; the right bar represents the average of four subjects. *shows significant difference at each 24h period from the first 24h period. *p<0.05, **p<0.01, ***p<0.001

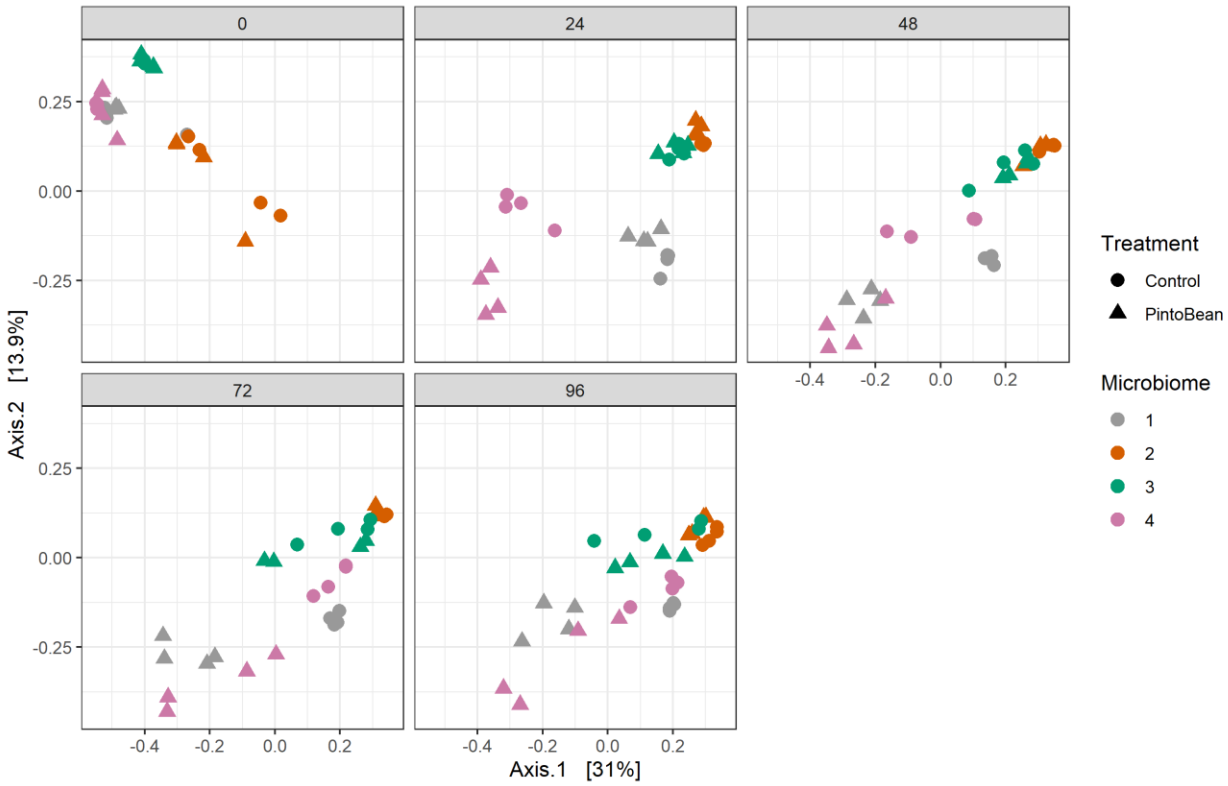

Figure 3.3 β-diversity, principal coordinates analysis (PCoA) biplot calculated based on Bray-Curtis distance among samples grouped by time, 0 h, 24 h, 48 h, 72 h, 96 h.

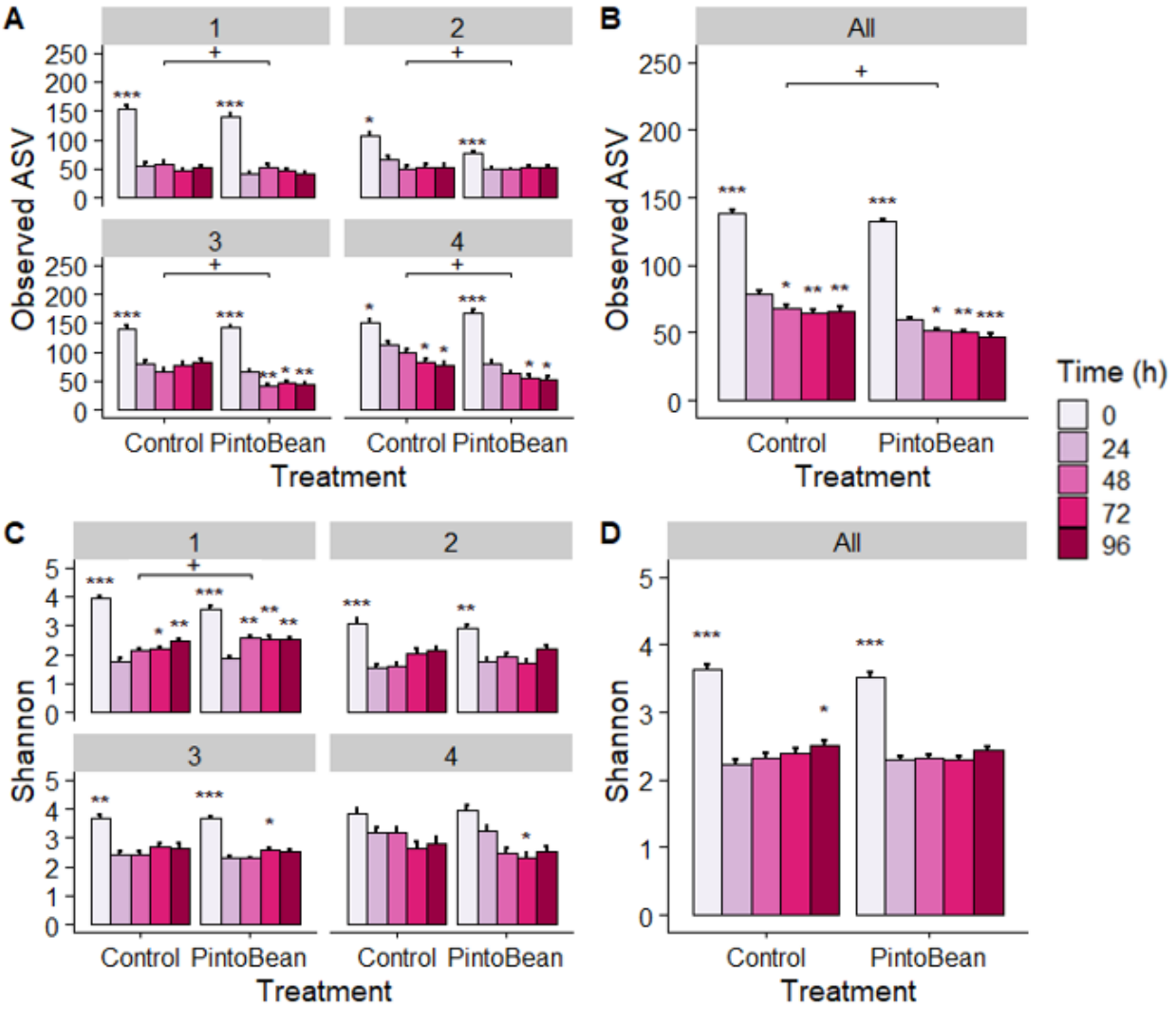

Figure 3.4 α-Diversity - Observed Amplicon Sequence Variances (ASVs) index during each 24 hour period by microbiome (A) and across all microbiomes (B), Shannon-index during each 24 hour period by microbiome (C) and across all microbiomes (D). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.05 for the comparison between treatments, Control and Pinto Bean (Dunnett's test).

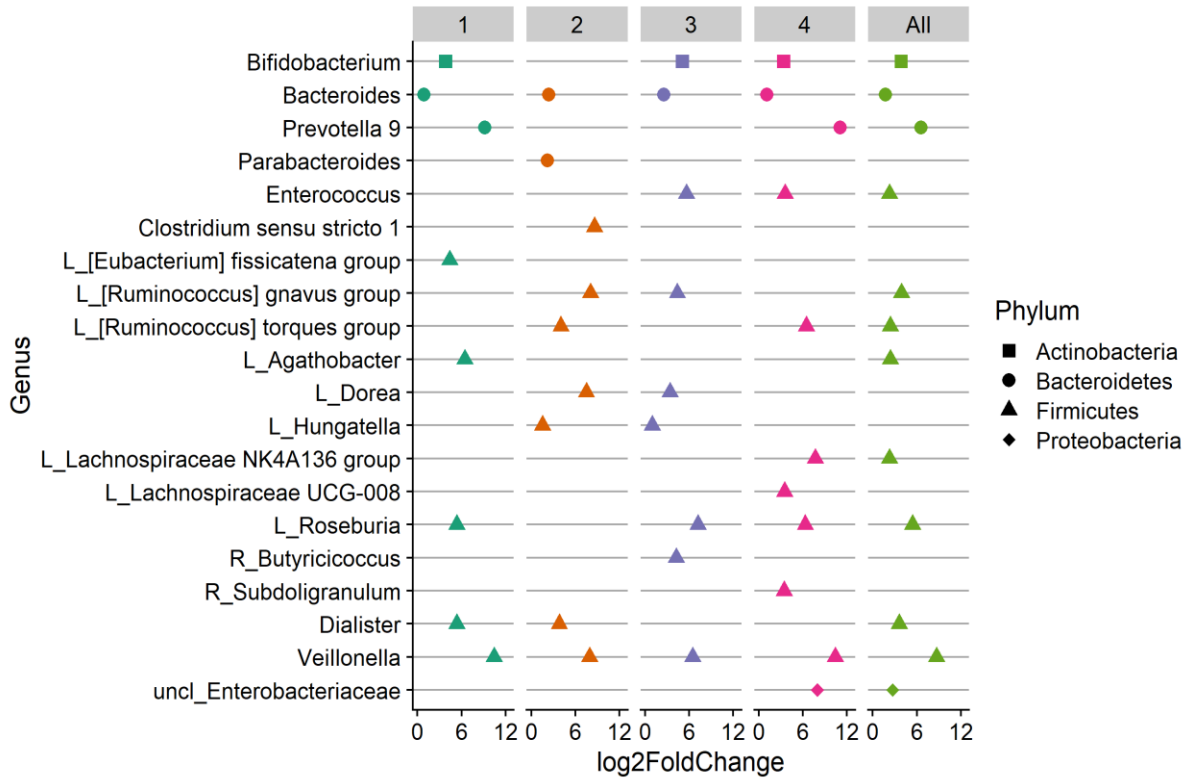

Figure 3.5 Genera with higher abundance in pinto bean treated samples compared with control after 96 h of fermentation. Subpanels represent the results from each of the four microbiomes and from all microbiomes together. The L or R prefix before some genera indicates the family to which that genus belongs, *Lachnospiraceae* or *Ruminococcaceae*, respectively.

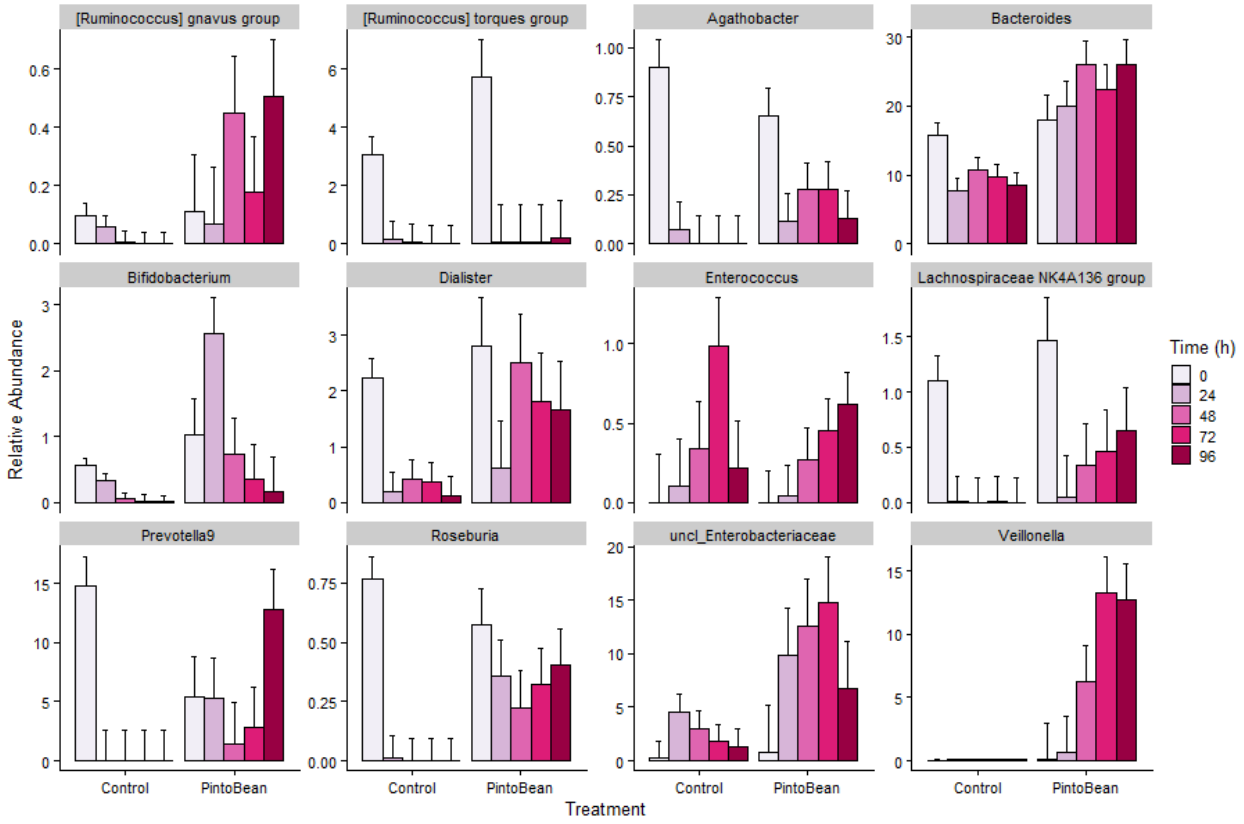

Figure 3.6 The relative abundance of genera *[Ruminococcus]gnavus* group, *[Ruminococcus]torque* group, *Agathobacter*, *Bacteroides*, *Bifidobacterium*, *Dialister*, *Enterococcus*, *Lachnospiraceae* NK4A136 group, *Prevotella* 9, *Roseburia*, uncl_*Enterobacteriaceae*, and *Veillonella* across all microbiomes during each 24 h.

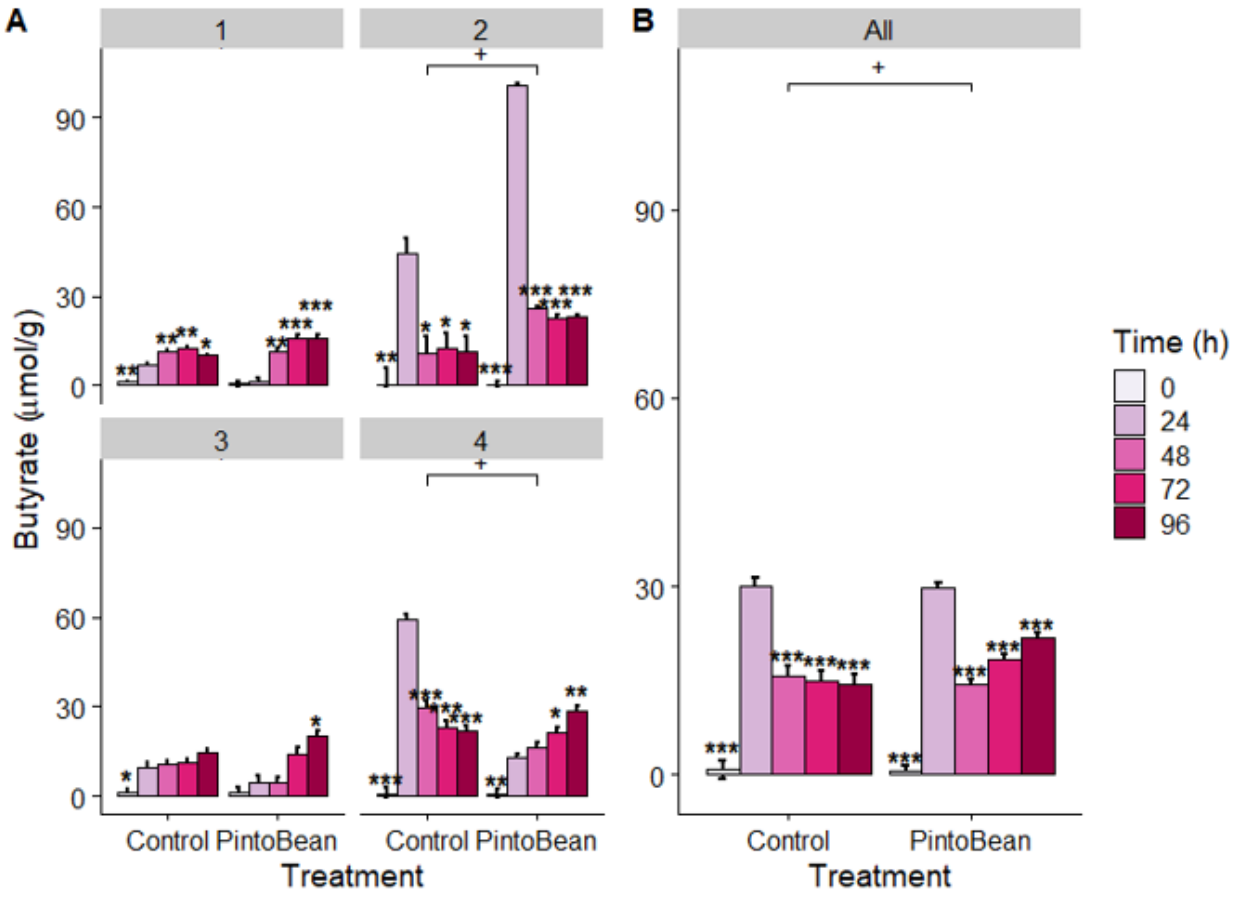

Figure 3.7 Butyrate production during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.05 for the comparison between treatments, Control and Pinto Bean (Dunnett's test).

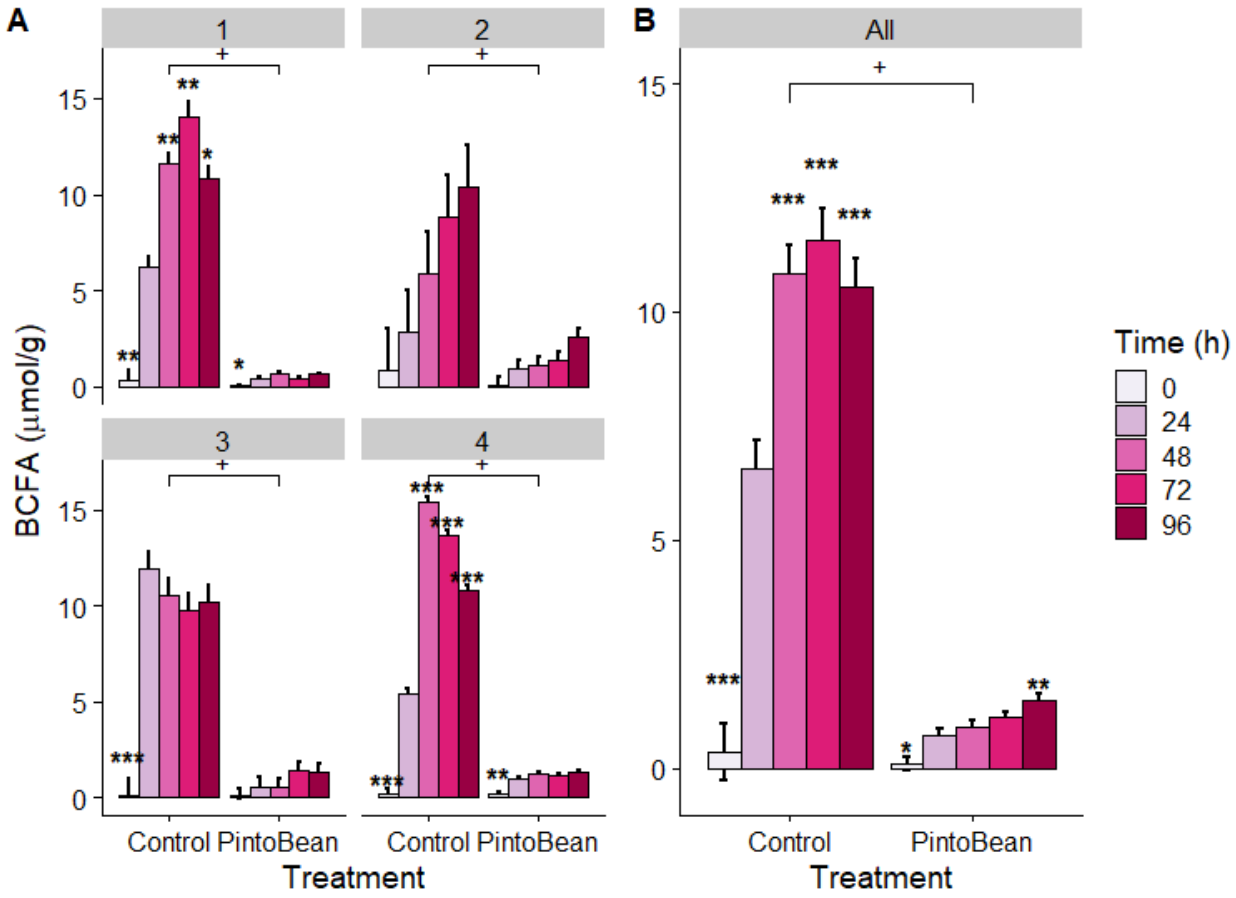

Figure 3.8 Total branched chain fatty acid (isobutyrate, isovalerate) production during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.001 for the comparison between treatments, Control and Pinto Bean (Dunnett's test).

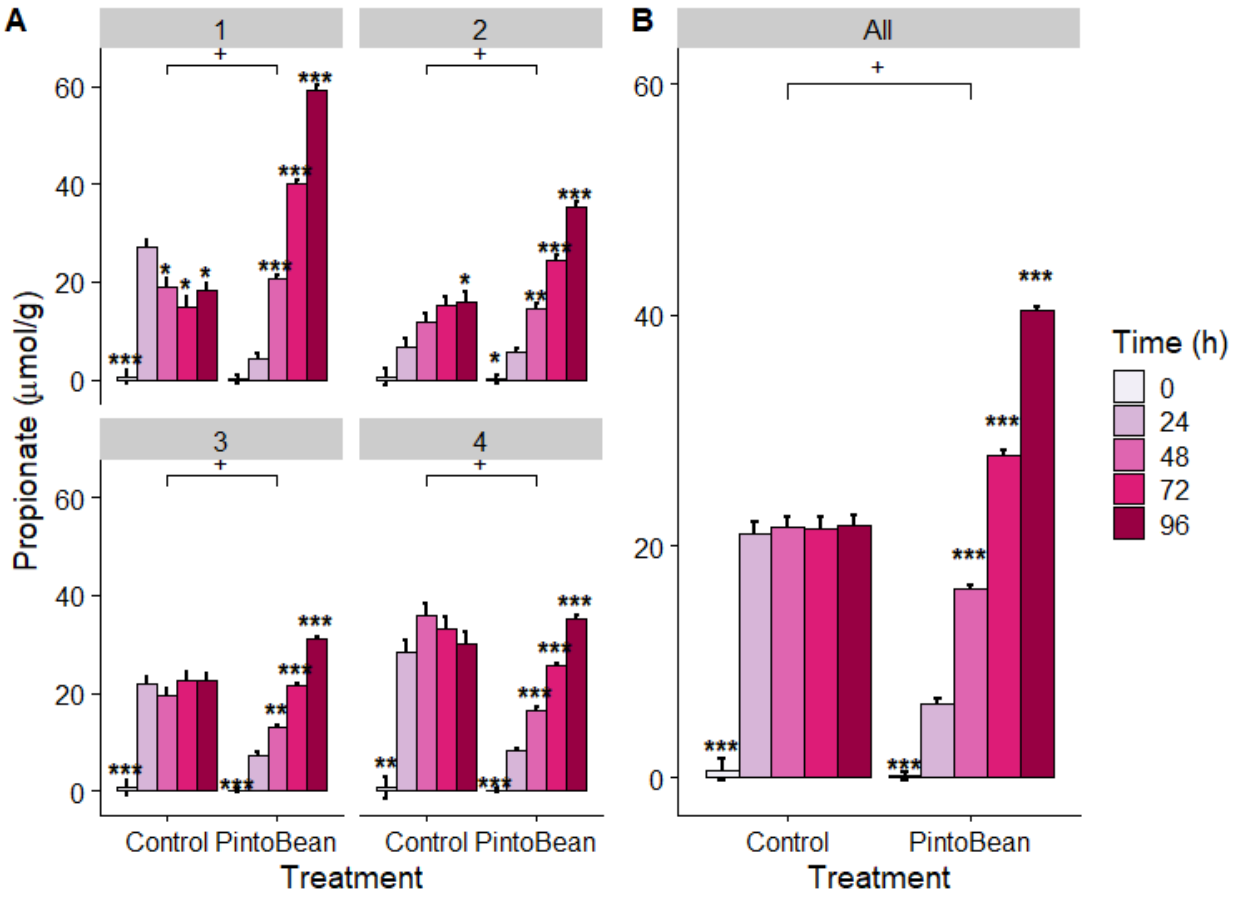

Figure 3.9 Propionate production during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.05 for the comparison between treatments, Control and Pinto Bean (Dunnett's test).

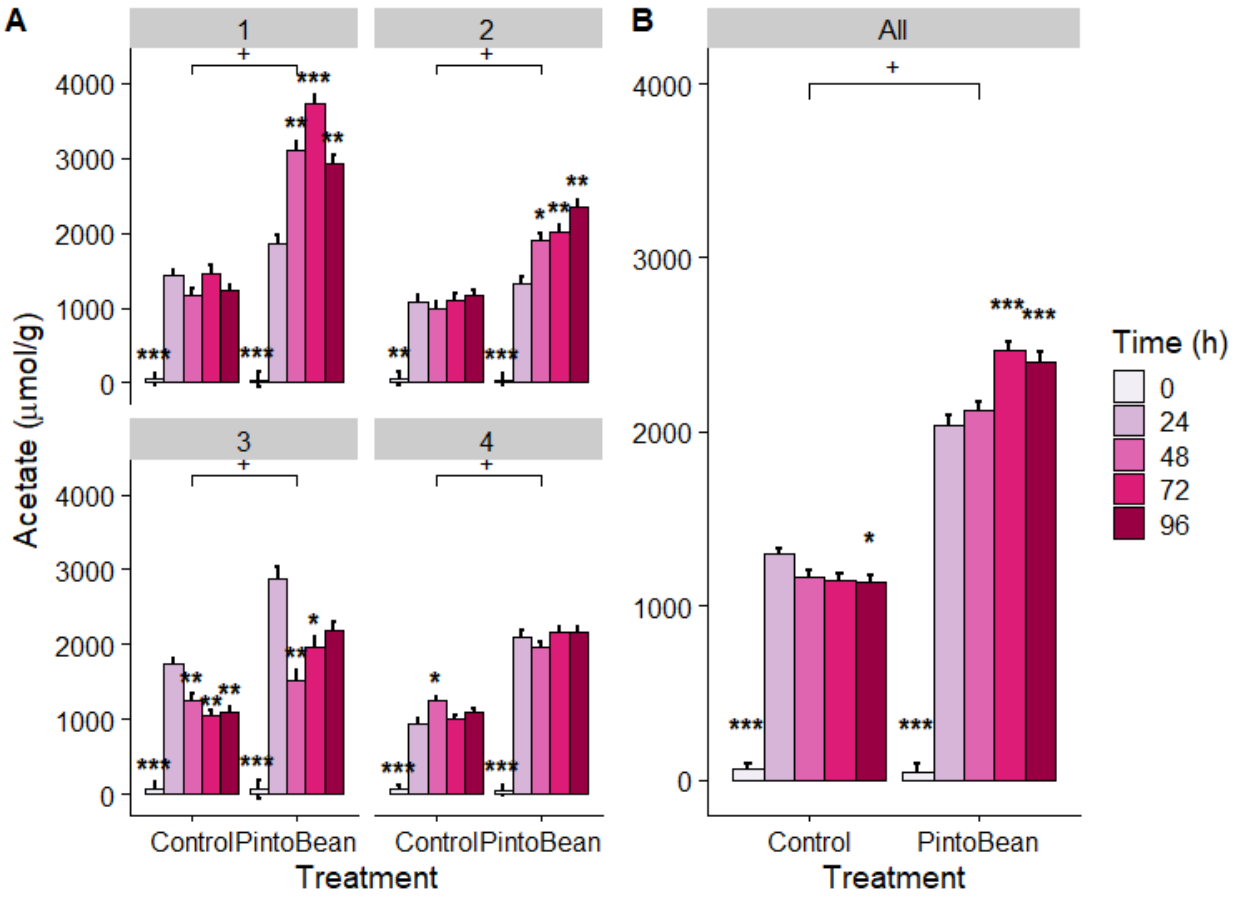

Figure 3.10 Acetate production during each 24 hour period by microbiome (A) and across all microbiomes (B). Error bars show standard error; *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.0001 for the comparison between treatments, Control and Pinto Bean (Dunnett's test).

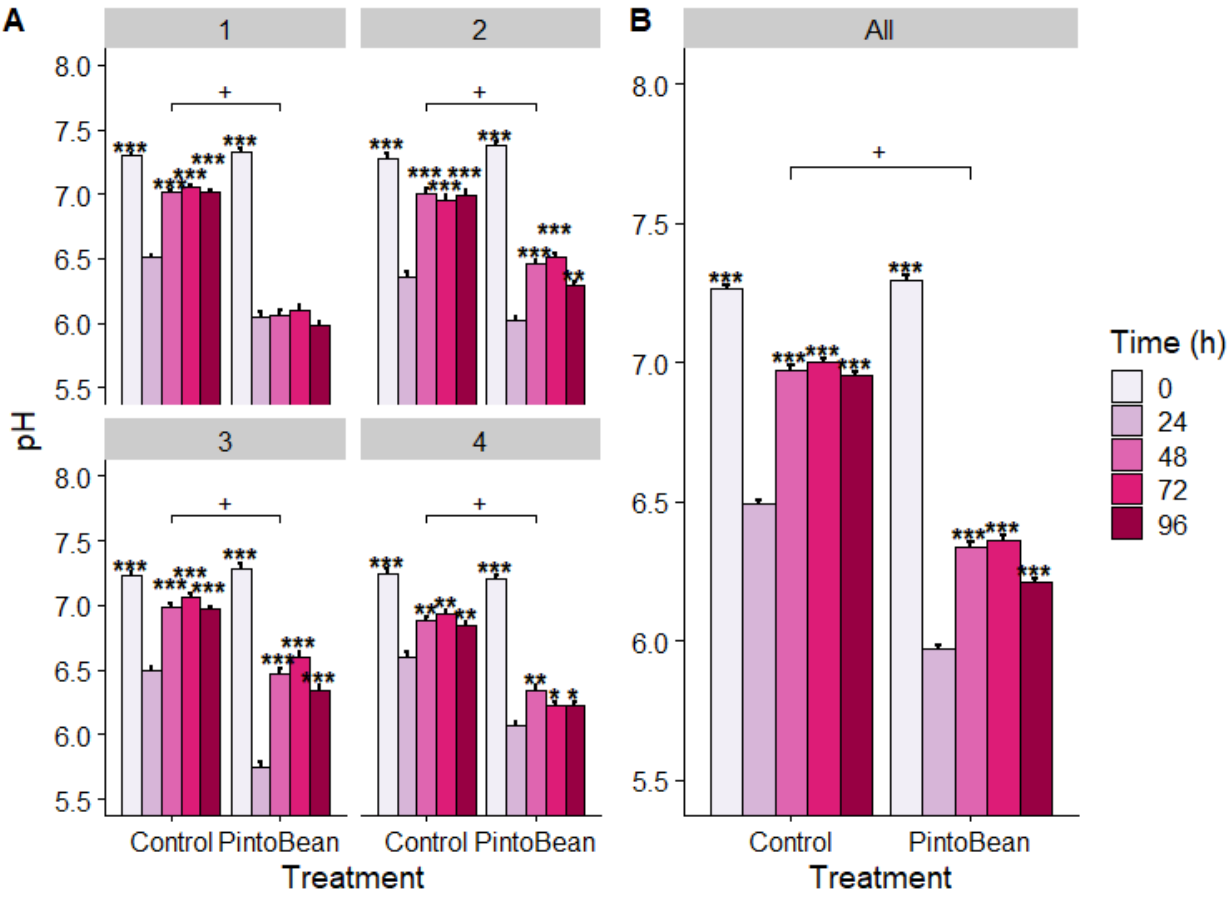

Figure 3.11 The pH value of consortia in control group and pinto bean group during each 24 hour period by microbiome (A) and across all microbiomes (B). *shows significant difference at each 24h period from the first 24h period. *p<0.05, **p<0.01, ***p<0.001 for the comparison with the corresponding 24 h sample (Dunnett's test). + p<0.0001 for the comparison between treatments (control group and pinto bean group) with Dunnett's test.

CHAPTER 4 GENERAL CONCLUSIONS

4.1. Conclusions

This thesis builds on literature regarding the characteristic and functionality of wheat bran (WB)-associated microbiomes and pinto bean (PB)-associated microbiomes. As different food sources especially dietary fiber are known to impact the gut microbial composition, a group of key microbes was hypothesized to be associated with the utilization of WB- non-digestible carbohydrates (NDC) and PB-NDC, respectively. At the end of 96 h of fermentation, despite interindividual differences, a total of 10 genera including 2 from *Lachnospiraceae* and 1 from R*uminococcaceae* were elevated across all WB-associated microbiomes. Similarly, despite with interindividual differences, there were a total of 12 genera including 5 from *Lachnospiraceae* characterized across all PB treated fecal microbiomes. The WB- associated microbiomes had a strong response to *Prevotella* 9 while PB- associated microbiomes had a strong response to *Bacteroides.*

My original intent was to identify WB- and PB-associated microbial communities with high capacity to ferment the dietary fibers in these substrates. I was able to identify such a community with the WB, where the ability of the microbiomes to ferment the dietary fibers from wheat bran increased over 96 h of fermentation. However, this did not occur for the PB-associated microbiotas. Perhaps the difference in solubility of dietary fibers in WB and PB was the reason for the different outcomes observed. The modification introduced to the stepwise *in vitro* fermentation procedure was designed to favor only the microbes that attach to and utilize insoluble NDC. WB NDC are almost all insoluble (~98%) (Arcila et al., 2015), while PB NDC consist of about 25% soluble NDC (Felker et al., 2018). Therefore, for the WB experiments, I

selected for microbes that were capable of utilizing nearly all of the NDC, while in the PB experiments I may have selected for microbes that were capable of utilizing only about 75% of the total NDC; the ability of the microbiome to ferment the soluble NDC was lost. Thus, in future work, it may be interesting to repeat the experiment, but this time differentiating between soluble and insoluble NDC to confirm if this explanation is true.

In another follow-up study, the WB- or PB-associated microbiomes (after 96 h of fermentation) could be spiked into fecal microbiomes with low NDC utilization capabilities. The purpose of this study would be to determine if the WB- or PB-associated microbiomes identified in this thesis can increase NDC utilization in fecal microbiomes. This could be a strategy to change the NDC utilization capability of a microbiome.

4.2. References

1. Arcila, J. A., Weier, S. A., & Rose, D. J. (2015). Changes in dietary fiber fractions and gut microbial fermentation properties of wheat bran after extrusion and bread making. *Food Research International*, *74*, 217-223.

2. Felker, F. C., Kenar, J. A., Byars, J. A., Singh, M., & Liu, S. X. (2018). Comparison of properties of raw pulse flours with those of jet-cooked, drum-dried flours. *LWT*, *96*, 648-656.