CONSEQUENCES OF DIETARY FIBERS AND THEIR PROPORTION ON THE FERMENTATION OF DIETARY PROTEIN BY HUMAN GUT MICROBIOTA

by

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Thank you for teaching me something new every day and filling my life with humor, love, and fantastically awesome chaos.

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I came to Purdue Food Science as an undergraduate with the intention of learning everything about starch structure and chemistry so that I could improve upon my gluten free recipes. Afterwards, as luck would have it, I was looking to get my master's degree at the same time Dr. Bruce Hamaker was looking for a gluten free baking expert. So, first and foremost, I want to thank Dr. Hamaker for his support and guidance and, in particular, for providing a quiet, reassuring confidence in me when I felt lost and overwhelmed.

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ABSTRACT

In the human gut, bacterial fermentation of dietary fibers and proteins produces metabolites, primarily as short-chain fatty acids (SCFA), that are highly beneficial for host health. However, unlike dietary fiber, bacterial fermentation of protein additionally generates potentially toxic substances such as ammonia, hydrogen sulfide, amines, and indoles. It is believed that most gut bacteria favor utilization of dietary fiber over that of protein for energy. Therefore, when fermentable dietary fiber is readily available to colonic bacteria, protein fermentation, and its subsequent potentially toxic metabolites, remains relatively low. Dietary intake primarily determines the quantity of dietary fiber and protein substrate available to the gut microbiota and the resulting profile of metabolites produced. Increased protein consumption is associated with deleterious health outcomes such as higher risk of colorectal cancer and type II diabetes. Conversely, diets following US dietary recommendations are high in fiber, which promote a healthy microbiome and are protective against disease. Diets following the recommendation are also moderate in protein intake so that, ultimately, far more fiber than protein is available for colonic bacterial fermentation. On the contrary, dietary fiber intake is chronically low in a standard Western diet, while protein consumption is above dietary recommendations, which results in nearly equal amounts of dietary fiber and protein available for gut microbial fermentation. Furthermore, the popularity of high-protein diets for athletes, as well as that of high-protein low-carbohydrate diets for weight loss, may flip fiber and protein substrate proportions upside down, resulting in more protein than fiber available in the gut for fermentation. The objective of this study was to elucidate how substrate ratios in protein-fiber mixtures affect protein fermentation and metabolites, as well as examine the degree to which fiber source may influence these outcomes. Each dietary fiber source [fructooligosaccharides (FOS), apple pectin (Pectin), a wheat bran and raw potato starch mixture (WB+PS), and an even mixture of the three aforementioned fibers (Even Mix)] and protein were combined in three ratios and provided as substrate for *in vitro* fecal fermentation to understand how low, medium, and high fiber inclusion levels influence fermentation outcomes. They were compared to 100% protein and fiber (each different fiber) controls. Branched-chain fatty acids (BCFAs), metabolites produced exclusively from protein fermentation, were used as a measure of protein fermentation; the data were normalized based on the initial quantity of protein within the substrate. In protein-fiber substrate mixtures, only FOS

and Even Mix inhibited BCFAs (mM/g protein basis) and only when they made up at least half of the substrate. Unexpectedly, the rate of protein fermentation was increased when the protein-fiber substrate contained 25% WB+PS fiber, possibly due to the starch component of the fiber. There was evidence that when pH drops during fermentation, as was the case for protein-FOS mixtures, it played a significant role in suppressing protein fermentation. Ammonia production was not largely affected by increasing the proportion of dietary fiber. A significant reduction did not occur until FOS made up at least 50% of the protein-fiber substrate; for Pectin, WB+PS, and Even Mix fibers, 75% inclusion was required for a significant decrease in ammonia. Interestingly, protein was butyrogenic. Protein as the sole substrate produced more butyrate than either Pectin or Even Mix as the sole substrates, and in fact, addition of Pectin to protein significantly reduced butyrate concentrations. However, the possible benefits of butyrate produced via protein fermentation needs to be tempered by the production of potentially toxic compounds and the association between protein fermentation and colorectal cancer. Overall, the thesis findings showed protein fermentation to be relatively stable and not easily influenced by increasing the availability of dietary fiber, and no clear evidence of microbial preference for carbohydrates over protein was found.

CHAPTER 1. INTRODUCTION

On a Western diet, nearly equal amounts of proteins and dietary fibers are delivered to the large intestine daily (Chacko & Cummings, 1988; U.S. Department of Agriculture, Agricultural Research Service, 2018), where they can be metabolized by the approximately 38 trillion bacterial cells present (Sender, Fuchs, & Milo, 2016). Bacterial fermentation of carbohydrates and proteins[1] produces metabolites, primarily as short-chain fatty acids (SCFA), which are highly beneficial for human health. However, protein fermentation also generates branched-chain fatty acids (BCFA) and potentially toxic substances; a situation that does not occur from carbohydrate fermentation. Collectively, these bacterial metabolites can be absorbed through the gut epithelium and transported throughout the body to influence the health or disease-state of multiple body systems (McNeil, Cummings, & James, 1978).

Diet significantly impacts the metabolites produced by the gut microbiota. Most gut bacteria favor carbohydrates, such as dietary fibers, for energy over proteins (Macfarlane, Gibson, & Cummings, 1992). Therefore, it is believed that when fermentable dietary fibers are readily available, bacterial protein fermentation and its production of potentially toxic products remain relatively low. On diets following US dietary recommendations, far more fiber than protein is available for colonic bacterial fermentation. Diets such as these are high in dietary fiber, which promote a healthy microbiome and is protective against disease. However, on a standard Western diet, the substrate proportions presented to gut microbiota are drastically different with protein being approximately equal to dietary fiber. Furthermore, the popularity of high-protein diets for athletes, as well as that of high-protein low-carbohydrate diets for weight loss, may flip fiber and protein substrate proportions upside down, giving more protein than fiber available for fermentation in the gut. Numerous studies have been conducted on the effect of dietary fiber and fat intake levels on gut microbiome health and disease, but comparatively little exists for that of protein intake levels. Yet, protein intake levels significantly affect gut microbial fermentation outcomes. Increased protein consumption is associated with dysbiosis and deleterious health outcomes such as increased risk

[1] Throughout the text in the context of fermentation, the word ‘protein’ is used for simplification to encompass proteins, peptides, and amino acids collectively unless otherwise specified.

for type II diabetes (Sluijs et al., 2010) and colorectal cancer (Armstrong & Doll, 1975; International Agency for Research on Cancer, 2015; Scheppach, W. et al., 1999).

Very few studies have been conducted on the effect of radically different protein and dietary fiber proportions available to the gut microbiota, such as that which can occur with the three diets mentioned above. A shift in the ratio of carbohydrates and proteins available to microbiota is likely to result in a different profile of fermentation products since microbiota vary in metabolic capabilities, pathways, and output. Insight into how differing amounts of protein and carbohydrates affect the quantity and type of fermentation products, both beneficial and potentially toxic, could lead to improved dietary recommendations. Furthermore, the type of available fiber likely also influences these differences, with some fibers potentially stifling protein fermentation, which could be used to suppress toxic fermentation products and lead to improved gut health.

1.1 Research Objectives and Design Explanation

The presence of both fermentable carbohydrates and proteinaceous substances are ubiquitous in the large intestine (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987), yet the interaction effects of simultaneous protein and dietary fiber fermentation in the gut has not been well studied. Populations consuming an average Western diet are chronically low in dietary fiber consumption, averaging 17.3 grams per day[2], and above recommended intakes for protein, with an average daily protein intake of 69.4 grams[2] (Berryman, Lieberman, Fulgoni, & Pasiakos, 2018; FAO/WHO/UNU, 2007; Hoy, M.K. & Goldman, J.D., 2014; U.S. Department of Agriculture, Agricultural Research Service, 2018; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). Based on these estimates, the result is an approximately equal amount of protein and dietary fiber available daily for fermentation in the large intestine (Bax et al., 2013; Chacko & Cummings, 1988; Gaudichon et al., 2002; K.R. Silvester & Cummings, 1995). Bacterial metabolism of proteins produces potentially toxic metabolites such as ammonia and hydrogen sulfide that may negatively affect human health (Macfarlane & Allison, 1986). Dietary fibers, which release beneficial metabolites when fermented in the gut, could be leveraged to counteract potentially toxic protein fermentation products.

[2] Based on males and females in the United States aged 20 and over (U.S. Department of Agriculture, Agricultural Research Service, 2018)

For this study, our goals were two-fold. Firstly, to determine how the substrate proportions of dietary protein and fiber affect the type and quantity of metabolites produced. Secondly, to leverage known fermentation characteristics of specifically chosen dietary fibers to produce high concentrations of beneficial SCFAs and inhibit the production of protein fermentation metabolites.

Every day, mixtures of undigested dietary proteins and fibers enter the large intestine. The ratio of proteins and fibers in this mixture is largely based on dietary intake. Similarly, in this study, dietary protein-fiber mixtures were provided as substrate for *in vitro* fecal fermentation to achieve our primary objective of characterizing the effects of protein to fiber ratio and dietary fiber source on protein fermentation and its byproducts. Our secondary aim was targeted amelioration and/or suppression of protein fermentation products through the utilization of specific fibers.

We chose three ratios of protein to fiber to understand how low, medium, and high fiber inclusion levels influence fermentation outcomes in a protein-fiber substrate mixture. These levels effectively mimicked the estimated protein to fiber ratios entering the gut of a person consuming an average Western diet (50% protein, 50% fiber), a diet based on governmental health organization recommendations (25% protein, 75% fiber), or that of a high-protein diet typically used by athletes or for weight loss (75% protein, 25% fiber).

Fermentation metabolites vary by protein source. Based on average American dietary consumption of 85% animal-based and 15% plant-based protein, we used a similar mixture of proteins to simulate the *in vivo* environment as closely as possible.

Finally, since a broad understanding of metabolite changes was the primary goal, a homogenate of fecal donations was used as the *in vitro* fermentation inoculum and measurements were recorded after 24 and 48 hour incubations.

1.2 Hypothesis

We hypothesized specific fiber sources, at certain incorporation levels, could be used to ameliorate protein fermentation and/or BCFAs and ammonia, both products of protein fermentation.

Furthermore, it was hypothesized that the concentration of BCFAs and ammonia would decrease as the ratio of fiber to protein increased. It was reasoned that increasing levels of fiber would result in higher concentrations of beneficial SCFAs. Two single-source dietary fibers and two dietary fiber mixtures were selected based on structural and fermentative properties for a targeted increase or decrease of specific by-products of carbohydrate or protein fermentation (further outlined below). The fibers chosen were fructo-oligosaccharides (FOS), apple pectin (Pectin), a 50/50 mixture of wheat bran and raw potato starch (WB+PS), and an even mixture of the 3 aforementioned fibers (Even Mix).

1.2.1 Detailed Fiber Source Hypotheses

FOS is a soluble non-viscous fiber that is quickly, easily, and efficiently fermented primarily in the proximal colon due to its simple repeating structure. This efficient fermentation produces voluminous SCFAs in a relatively short period of time and lowers the environmental pH. When the pH is decreased, peptide and amino acid fermentation are inhibited, subsequently suppressing formation of BCFAs (Smith & Macfarlane, 1998; Walker, Duncan, Leitch, Child, & Flint, 2005). We therefore hypothesized that when presented in a mixture with protein, bacterial preference for FOS over the more complex metabolic process for protein utilization would result in the fast fermentation of FOS, quickly generating SCFAs that significantly lower the pH, and ultimately inhibit protein fermentation.

Like FOS, pectin is also a soluble fiber that is completely fermentable. However, pectin is a viscous fiber with a more complex structure that has been shown to be slower fermenting and lead to the production of SCFAs in more distal regions of the colon (Ferreira-Lazarte, Moreno, Cueva, Gil-Sánchez, & Villamiel, 2019). Studies have shown pectin supplementation promotes healthy bacterial groups that are decreased by protein fermentation such as *Bifidobacterium* spp, *Bacteroides* spp, *Faecalibacterium prausnitzii*, and *Prevotella* (Bang et al., 2018; Ferreira-Lazarte et al., 2019; Mu, Yang, Luo, & Zhu, 2017). Additionally, pectin fermentation has resulted in increased acetate and butyrate (Bang et al., 2018; Chen, 2016; Tian et al., 2017), and a recent study found pectin to decrease ammonium concentrations in the colon (Ferreira-Lazarte et al., 2019). We hypothesized pectin would suppress ammonia formation and promote bacteria associated with increases of butyrate and acetate production.

A mixture of half wheat bran and half raw potato starch (WB+PS) was chosen for its butyrogenic properties and slow rate of fermentation. Wheat bran fiber is largely insoluble and composed primarily of a matrix of arabinoxylan, cellulose, and beta-glucan fibers (Maes & Delcour, 2002; Stevenson, Phillips, O'Sullivan, & Walton, 2012). In the first 12 hours of *in vitro* fermentation, wheat bran can produce butyrate at levels similar to FOS, after which time it produces far greater amounts of butyrate than FOS. It also outperforms the butyrate production of corn, sorghum, and rice brans (Tuncil, Thakkar, Arioglu-Tuncil, Hamaker, & Lindemann, 2018). Raw potato starch is a type II resistant starch consisting of glucose polymers resistant to digestion due to physical inaccessibility. It has been found to be butyrogenic due to its acetogenic behavior, as acetate can be converted by some bacteria to butyrate, as well as to decrease BCFAs (Mathers, Smith, & Carter, 1997; Mentschel & Claus, 2003). A synergistic effect exists when combining wheat bran and resistant starch. A human diet study found that the combination increased butyrate, lowered fecal pH, and also lowered concentrations of the protein fermentation products ammonia and phenols (Muir et al., 2004). Its use in our study was hypothesized to reduce protein fermentation and ammonia concentrations for a longer period than a more quickly fermented fiber. Additionally, it was included for its butyrogenic properties because butyrate has an anti-inflammatory effect which could counteract the potentially inflammatory effects of some protein fermentation products such as ammonia and hydrogen sulfide.

Finally, an even mixture of the three fibers, FOS, pectin, and WB+PS, was chosen. There is evidence that fiber mixtures ferment more slowly than their individual components (Tuncil et al., 2017). We hypothesized a longer, slower fermentation period would result in a longer-sustained suppression effect on protein fermentation due to microbial preference for carbohydrate energy sources over that of protein.

CHAPTER 2. LITERATURE REVIEW

The bacterial fermentation of substrates such as dietary fibers and proteins in the large intestine plays a vital role in human health. Many of the metabolites produced as a result of fermentation [i.e., short chain fatty acids (SCFA)] beneficially regulate many biochemical and physiological processes in the colon and throughout the body. However, some metabolites are implicated with deleterious outcomes. Whether more beneficial or deleterious metabolites are produced is largely determined by diet since it consequently determines the substrate availability to the microbiota in the large intestine. Most gut bacteria favor carbohydrates, such as dietary fibers, for energy over proteins (Macfarlane, Gibson, & Cummings, 1992). When high levels of dietary fibers and other carbohydrates are readily fermented, bacterial protein fermentation and its potentially toxic byproducts remain relatively low.

When the dietary recommendations set by most governments are followed, far more dietary fiber than protein is available for colonic bacterial fermentation. Dietary fiber has a protective effect against disease (Dahl & Stewart, 2015; Desai et al., 2016; Llewellyn et al., 2018; World Cancer Research Fund/American Institute for Cancer Research, 2018b), reduces the risk of colorectal cancer (World Cancer Research Fund/American Institute for Cancer Research, 2018b), as well as lowers the risk for other gastric cancers, obesity, type II diabetes, and cardiovascular disease (Dahl & Stewart, 2015). This is due in part because dietary fibers are fermented by beneficial gut bacteria for energy. This sustains and promotes their growth, in turn producing greater and greater amounts of SCFAs which are vitally important for maintaining and restoring health in humans (Ríos-Covián et al., 2016). Both increased presence of these commensal bacteria and SCFAs help to inhibit growth of pathogenic microbiota and lower protein fermentation activity.

However, in the United States and many other industrialized countries, average protein consumption exceeds dietary recommendations, while dietary fiber intake falls short (Berryman et al., 2018; FAO/WHO/UNU, 2007; Hoy, M.K. & Goldman, J.D., 2014; U.S. Department of Agriculture, Agricultural Research Service, 2018; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). Compared to a healthy diet, this shifts the proportions of protein and carbohydrate substrates available for fermentation by gut bacteria, thereby

increasing protein fermentation metabolites produced with potentially toxic consequences. Increased protein consumption is associated with dysbiosis and negative health outcomes (Blachier et al., 2019; Blachier, Mariotti, Huneau, & Tomé, 2007; Duncan et al., 2007; Fan et al., 2015; Gilbert, Ijssennagger, Kies, & van Mil, 2018; Liu et al., 2014; Mu et al., 2017; Richardson, McKain, & Wallace, 2013; Russell et al., 2011; Katherine R. Silvester, Bingham, Pollock, Cummings, & O'Neill, 1997; Toden, Bird, Topping, & Conlon, 2007; Windey, De Preter, & Verbeke, 2012). Protein fermentation may be deleterious to gut health through several mechanisms such as inducing inflammation (Lan et al., 2015) and thinning the colonic mucus barrier (Toden et al., 2007). Furthermore, as shown in Figure 2.1, the consumption of animal protein, particularly red and processed meats, is positively correlated with colorectal cancer (Armstrong & Doll, 1975; International Agency for Research on Cancer, 2015; Scheppach, W. et al., 1999). Not coincidentally, a higher proportion of colorectal cancer and protein fermentation occurs in the distal colon.

2.1 Proteins and Carbohydrates in the Large Intestine: Origin and Consequences

During digestion, dietary proteins and carbohydrates are first exposed to α-amylase in the mouth; α-amylase begins the process of breaking down starch. Once in the stomach, gastric acid denatures and partially unfolds dietary proteins, while pepsin, the gastric protease in the stomach, hydrolyzes some proteins into polypeptides. In the small intestine, a number of enzymes work to hydrolyze carbohydrates into monosaccharides and proteins into small peptides and amino acids. Monosaccharides and amino acids diffuse across the epithelial cell membranes where they enter the capillary blood in the villi and are transported to the liver (Frayn, 2010). However, not all carbohydrates and proteins are completely broken down and absorbed in the small intestine. These incompletely digested carbohydrates (composed primarily of dietary fibers[3]) and proteins are passed to the large intestine where they become available for bacterial fermentation.

[3] The official definition of dietary fiber in the U.S is, "non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units) determined by FDA to have physiological effects that are beneficial to human health. (U.S Food & Drug Administration, 2019)"

Whereas digestion from the mouth through the small intestine is rapid, taking approximately 6-8 hours, the process through the large intestine is much slower, taking 40 hours on average to go through the ascending, transverse, and finally the descending colon (Metcalf et al., 1987). Trillions of bacteria residing in the colon can metabolize the undigested fermentable carbohydrates and proteins as these substrates make their transit through the large intestine (Sender et al., 2016). Slower colonic transit times lead to increased protein fermentation (Cummings, Hill, Bone, Branch, & Jenkins, 1979; Muir et al., 2004; Smith & Macfarlane, 1996) which, as mentioned earlier, is negatively associated with health. Insoluble dietary fibers, on the other hand, increase transit time, which reduces protein fermentation.

2.2 Endogenous and Exogenous Protein Source Estimates

Dietary consumption provides most of the protein and peptides in the colon, while only a relatively small amount is derived from endogenous sources (Chacko & Cummings, 1988). On average, Americans consume a moderately high-protein diet, with an estimated 85 grams per day derived from animal sources and 15 grams per day of plant protein (Gardner, Hartle, Garrett, Offringa, & Wasserman, 2018; U.S. Department of Agriculture, Agricultural Research Service, 2018). Protein is 86 to 95% digestible in the small intestine of humans, with animal sources generally being more digestible than plant proteins (Bax et al., 2013). Despite being so highly digestible, an estimated 9-15 grams of dietary protein per day enters the adult human colon when on a moderate protein diet (Chacko & Cummings, 1988; Cummings & Macfarlane, 1997; Gaudichon et al., 2002). However, increased protein intakes have been found to coincide with increased protein delivery and toxic protein fermentation byproducts in the large intestine (Cummings et al., 1979; Duncan et al., 2007; Russell et al., 2011; K.R. Silvester & Cummings, 1995). In addition to protein from host diet, a relatively small amount from endogenous sources[4] such as sloughed epithelial cells, mucin, and digestive enzymes is delivered daily to the colon. A study using ileostomy subjects found that even when protein was only 1% of energy intake 3 grams of protein escaped the terminal ileum on average (Chacko & Cummings, 1988). Different measurement methods in other studies have found higher endogenous protein flows closer to 5 grams per day (Deglaire, Bos, Tomé, & Moughan, 2009; Gaudichon et al., 2002; Moughan, Butts, Rowan, & Deglaire, 2005). To

[4] Bacterial protein is also often measured as an endogenous protein source, however this is not uniform across all studies.

summarize, approximately 12-18 grams of proteins and peptides are available daily for colonic microbial fermentation when on a moderate protein diet; this number is likely higher for those on a moderately high- or high-protein diet.

2.3 Colonic Bacterial Utilization of Protein

2.3.1 The Occurrence of Proteolysis in the Large Intestine

In the late 1980's and early 1990's, several studies using sudden death victims established and confirmed much of the foundational gut microbiome knowledge applicable to our current work. More specifically these studies demonstrated the following: (1) proteolysis occurs in the colon and bacteria are primarily responsible for its occurrence (Macfarlane, Cummings, & Allison, 1986), (2) bacterial fermentation of carbohydrates and proteinaceous sources occurs throughout the colon (Cummings et al., 1987), (3) significant amounts of protein and ammonia are found in all regions of the colon (Macfarlane, Gibson, & Cummings, 1992), (4) there is proportionally more protein fermentation in distal regions while fermentation of carbohydrates occurs more so in proximal regions (Macfarlane, Gibson, Beatty, & Cummings, 1992), and (5) protein fermentation is responsible for a much larger percentage of SCFA production in the distal colon than the proximal (Macfarlane, Gibson, Beatty, et al., 1992). In combination, these studies largely translated to an understanding that bacterial protein fermentation occurs in more distal regions of the large intestine due to microbial preference for carbohydrates over protein as an energy source. However, this statement can be somewhat misleading because protein fermentation is greater in the distal colon on a *proportional basis*, but it occurs throughout the large intestine (Cummings et al., 1987; Macfarlane, Gibson, Beatty, & Cummings, 1992). Quantitatively, there are more carbohydrates and proteins available in the proximal colon, which lead to more fermentative activity as a whole occurring in this region. Carbohydrate fermentation is responsible for a quantitatively larger proportion of SCFAs generated in proximal regions because, in addition to SCFAs (primarily acetate, propionate, and butyrate), the fermentation of proteins may produce BCFAs, ammonia, phenols, indoles, amines, and other non-volatile organic acids. Furthermore, specific enzymes are required for microbial protein/peptide/amino acid catabolism and fermentation that the majority of gut microbiota do not have. Many of these enzymes are inhibited in more acidic environments, which is created primarily by SCFA production, therefore making the proximal colon a

comparatively low pH environment due to the quantitatively higher production/accumulation of SCFAs in this region. Together this makes proteins generally slower fermenting in these regions. As the net substrate availability decreases distally, there is a consequent quantitative decrease in fermentative activity and therefore a rise in pH. The ratio of carbohydrates to proteins also decreases distally since more microbiota in the gut are capable of utilizing carbohydrates than proteins. The sum effect is that protein fermentation is responsible for a larger proportion of SCFAs generated distally. In the proximal colon it has been estimated that protein fermentation accounts for 17% of all SCFAs and 38% in the distal colon (Macfarlane, Gibson, Beatty, et al., 1992).

2.3.2 Structure of Proteins Delivered to the Gut

The majority of proteinaceous substrate available to microbiota in the large intestine arrives in the colon as either proteins (50%) or peptides (20-30%) (Chacko & Cummings, 1988), which are then hydrolyzed to peptides and amino acids by host or bacterial enzymes, and taken up by microbiota with specific transporters for synthesis or fermentation (Davila et al., 2013). It is believed that amino acids cannot be absorbed by the large intestinal epithelium (except in neonates) (van der Wielen, Moughan, & Mensink, 2017). However, amino acids may be taken up directly by gut bacteria for incorporation into bacterial protein structures; thus, amino acids are important for both bacterial growth and energy (Dai, 2011; Davila et al., 2013)

2.3.3 Gut Bacterial Catabolism of Proteins

Dietary proteins reaching the gut need to be further hydrolyzed for bacterial utilization; some bacteria are capable of taking up peptides while others can only take up amino acids. Proteolytic bacteria, capable of hydrolyzing proteins and peptides for energy metabolism, are diverse in the large intestine, ranging from primarily saccharolytic microbiota to obligate amino acid fermenters (Davila et al., 2013). Gut bacteria produce proteases to break peptide bonds, specifically, aspartic, cysteine, serine, and metallo proteases, but studies have shown that many more proteases come from human cells (Oliphant & Allen-Vercoe, 2019).

Microbial catabolism of amino acids begins by deamination or decarboxylation. Deamination produces carboxylic acid and ammonia, whereas decarboxylation produces amines and carbon

dioxide (Fan et al., 2015). High concentrations of SCFAs are produced when deamination is used by the gut microbiota (Fan et al., 2015; Portune et al., 2016). Subsequent steps of amino acid catabolism and products generated depend on the class of amino acid as shown in Figure 2.2. Most gut microbiota are capable of fermenting only specific amino acids. Using the gut contents of sudden death victims, *in vitro* growth experiments found that individual amino acids selected for different species of bacteria (Smith & Macfarlane, 1998). This signifies that the amino acid profiles of dietary proteins modulate the gut ecology and, in the future, could be harnessed to make specified changes within the gut environment.

2.4 Metabolites Produced via Gut Bacterial Fermentation of Proteins and Carbohydrates

The primary metabolites produced via bacterial fermentation of dietary fibers and proteins are the SCFAs acetate, propionate, and butyrate. Other metabolites include succinate, formate, lactate, ethanol, hydrogen, and carbon dioxide, many of which are intermediates that can later be converted to SCFAs via microbial cross-feeding activities (Russell, Hoyles, Flint, & Dumas, 2013). The fermentation of protein also generates BCFAs, primarily as iso-butyrate and iso-valerate, as well as ammonia, oxaloacetate, amines, phenolic and indolic compounds, hydrogen sulfide, and methane (Dai, 2011; Davila et al., 2013; Heimann, Nyman, Pålbrink, Lindkvist-Petersson, & Degerman, 2016; Richardson et al., 2013; Yao, Muir, & Gibson, 2016). The specific metabolites formed from protein fermentation depend on the available amino acids and the catabolic process utilized (Table 2.1). For example, metabolism of phenylalanine and tryptophan generates phenolic and indolic compounds (Dai, 2011), and hydrogen, carbon dioxide, and ammonia may be produced by the deamination of any amino acid (Davila et al., 2013).

On a bigger picture scale, proteinaceous metabolites in the large intestine are largely determined by dietary protein source and food processing which effect the profile and quantity of amino acids reaching the large intestine (Bax et al., 2013; Beaumont et al., 2017; Toden et al., 2007). Both amino acid composition and protein digestibility vary by food source and food processing (Windey et al., 2012). Animal proteins are typically more digestible than plant proteins. Plant proteins may be protected inside complex carbohydrate structures that host-endogenous enzymes are unable to hydrolyze in the small intestine. The cooking method of animal muscle proteins was shown to

change its digestibility and have a significant impact *in vitro* on bacterial composition and abundance as well as production of SCFAs and BCFAs (Shen, Chen, & Tuohy, 2010).

Collectively, colonic bacterial fermentation of dietary fibers and proteins produces extremely beneficial metabolites capable of modulating human health and disease on a systemic level. However, some metabolites of protein fermentation are implicated with negative effects on human physiology. Some of the most notable and primary metabolites of protein and fiber fermentation, are discussed in more detail below.

2.4.1 Short-Chain Fatty Acids

SCFAs are found in greatest proportion in the lumen of the proximal colon, produced primarily from the fermentation of carbohydrates and proteins, and quantitatively decrease towards the distal colon. Subsequently, the luminal pH is lowest in the proximal colon and increases towards the distal colon (Macfarlane, Gibson, & Cummings, 1992). In fecal samples provided by humans with a healthy microbiome, acetate was found in highest proportion, followed by propionate and butyrate (Russell et al., 2013). The molar ratio of these three SCFAs in fecal samples typically ranges between 3:1:1 to 10:2:1 (Rowland et al., 2018).

Generation of SCFAs from proteinaceous substrates is proportionally higher in the distal colon due to the depletion of available carbohydrate substrates and a higher pH. The specific SCFAs generated from protein fermentation depend on the chemical structure of the amino acids metabolized. Bacteria utilize glutamate and lysine to produce butyrate, alanine and threonine for propionate production, and acetate can be formed by glycine, alanine, threonine, glutamate, and lysine (Barker, 1981; Davila et al., 2013; Elsden & Hilton, 1978). Intestinal transit time, luminal pH, microbial composition, and availability of carbohydrates also affect the quantity and profile of SCFAs produced from proteinaceous substrates (Smith & Macfarlane, 1998). When compared to carbohydrates, studies have found fermentation of protein lowers the overall output of acetate, butyrate, and total SCFAs, while propionate remains comparable (Diether & Willing, 2019).

SCFAs lower the luminal pH, acting as an inhibitor of pathogenic bacteria, inhibiting proteolytic fermentation, and increasing mineral absorption. They can also serve as an energy source for

colonocytes, with butyrate being the primary source of energy for these cells. SCFAs are absorbed by the gut in a concentration-dependent manner and released via hepatic and portal venous systems (Layden, Angueira, Brodsky, Durai, & Lowe, 2013).

The most abundant SCFA is acetate which acts as a metabolite for the growth of bacterial cells in the gut, and is used in lipogenesis and cholesterol metabolism (Valdes, Walter, Segal, & Spector, 2018). Acetate may also help regulate appetite (Frost et al., 2014). Propionate promotes energy homeostasis by reducing hepatic glucose subsequently preventing weight gain (Chambers et al., 2015; De Vadder et al., 2014). Propionate has also been implicated in improving insulin sensitivity (Chambers et al., 2019). Butyrate is arguably the most important SCFA in regard to human health. It regulates inflammation, increases production of healthy colorectal cells, stimulates mucin production to help maintain the gut barrier, and stimulates production of healthy gut epithelial cells. It has also recently been found to enhance fat oxidation and reduce appetite via the gut-brain axis (Li et al., 2018). Both butyrate and propionate can prevent proliferation of colorectal cancer cells by inducing apoptosis (Rivière, Selak, Lantin, Leroy, & De Vuyst, 2016).

2.4.2 Branched-Chain Fatty Acids

The BCFAs (iso-valerate, iso-butyrate, and 2-methylbutyrate) are formed exclusively via the fermentation of branched-chain amino acids (leucine, isoleucine, and valine) and therefore, as mentioned previously, are an accurate indicator of proteolytic fermentation (Macfarlane, Gibson, Beatty, et al., 1992). Iso-valerate is usually the BCFA found in highest concentration. Based on most probable number (MPN) counts, 40% of gut bacteria capable of protein fermentation produce iso-butyrate, whereas a smaller proportion produce iso-valerate and 2-methylbutyrate (Smith & Macfarlane, 1998).

As with SCFAs, diet greatly influences the quantity and profile of BCFAs produced in the microbiome (Blachier et al., 2019; Russell et al., 2011). For example, high-protein diets were found to increase total BCFAs and the proportion of iso-valerate relative to iso-butyrate.

Studies with an objective to decrease toxic metabolites of protein fermentation often aim for suppression of BCFAs since they are an easily measured indicator of protein fermentation.

However, BCFAs are not considered to be toxic or potentially toxic byproducts of protein fermentation in the gut. BCFAs are poorly studied and very little is known about the effect they may have on host physiology. One study found that iso-butyrate may be used as an energy source by intestinal epithelial cells when butyrate is unavailable (Jaskiewicz et al., 1996). More recently, BCFAs were found to act similarly to SCFAs in their ability to modulate liver glucose and lipid metabolism (Heimann et al., 2016). There is also some indication that iso-butyrate may play a role in ionic regulation in the colon. One study found iso-butyrate to increase the diameter of isolated colonic crypts of rats by turning on the Na^+/H^+ exchanger (Diener, Helmle-Kolb, Murer, & Scharrer, 1993), while another study using a model of colonic epithelial cells found that iso-butyrate may regulate Na^+ absorption (Musch, Bookstein, Xie, Sellin, & Chang, 2001).

2.4.3 Ammonia

Ammonia is found at a fairly wide range of millimolar concentrations within the colon, from 3 to 44 mM, increasing towards the distal region (Wrong, Metcalfe-Gibson, Morrison, Ng, & Howard, 1965). The concentration in the colon is dependent on microbial deamination, microbial protein synthesis, and enterocyte metabolism. As previously stated, ammonia is produced via the deamination of amino acids as well as the hydrolysis of urea. In the large intestine the large majority of ammonia comes from fermentation of amino acids, whereas urea hydrolysis is only a very small minority. When ammonia is formed by the catabolism of amino acids, gut bacteria can use it directly for de novo protein synthesis (Diether & Willing, 2019).

Capable of being absorbed through the colonic mucosa (Wrong & Vince, 1984), ammonia is implicated in the disruption of several important processes within the gut. High concentrations of ammonia may inhibit butyrate uptake in intestinal epithelial cells, increase glycolysis in colonocytes, induce expression of pro-inflammatory genes in colonic intestinal epithelial cells, and lead to decreased intestinal barrier function (Blachier et al., 2019; Darcy-Vrillon, Cherbuy, Morel, Durand, & Duée, 1996; Davila et al., 2013; Vidal-Lletjós et al., 2017). Consuming more dietary fiber has been found to lower ammonia concentrations in the large intestine due to utilization by bacteria for nitrogen during periods of increased microbial growth (Birkett, Muir, Phillips, Jones, & O'Dea, 1996; Pieper et al., 2012; Katherine R. Silvester et al., 1997). Conversely, increased

dietary protein consumption increases the concentration of ammonia found in the lumen and in fecal output (Katherine R. Silvester et al., 1997).

2.4.4 Hydrogen Sulfide

Hydrogen sulfide (H_2S) gas is produced in the large intestine primarily by sulfate-reducing bacteria by sulfate reduction or by bacterial fermentation of sulfite, taurine, cysteine, methionine or sulfated organic compounds (Macfarlane, Gibson, & Cummings, 1992). Bacteria that utilize cysteine create ammonia and pyruvate in addition to H_2S. In the luminal environment of the large intestine, H_2S concentrations between 1.0 - 2.4 mM have been recorded (Macfarlane, Gibson, & Cummings, 1992). Fecal concentrations of H_2S increased substantially in subjects consuming a high-protein, high-meat diet (Magee, Richardson, Hughes, & Cummings, 2000), while fermentable dietary fibers suppressed H_2S formation *in vitro* (Yao et al., 2018).

H_2S is capable of permeating across the intestinal epithelium (Goubern, Andriamihaja, Nübel, Blachier, & Bouillaud, 2007), and when in excess it is a known mitochondrial poison inhibiting cellular respiration (Nicholls, 1975). H_2S can inhibit intestinal epithelial cell butyrate utilization, increase expression of pro-inflammatory cytokine expression, inhibit cellular respiration, and break down the mucous layer (Davila et al., 2013). A study using Chinese hamster ovary cells found it caused genomic DNA damage and suggested its implication in colorectal cancer (Attene-Ramos, Wagner, Plewa, & Gaskins, 2006). H_2S produced by gut microbiota is associated with ulcerative colitis (UC), Crohn's disease, and irritable bowel syndrome (Singh & Lin, 2015). For example, UC patients have elevated concentrations of H_2S (Nemoto et al., 2012), and sulfates can be used to induce a state similar to UC in rats (Leung et al., 2000). There is, however, some emerging evidence that H_2S is not solely a toxin but may also act as a mediator of inflammation, homeostasis, and repair in the gastrointestinal tract of humans (Wallace, Motta, & Buret, 2018). Compared to other tissues, intestinal epithelial cells were found to be extremely efficient at converting H_2S to thiosulfate (Furne, Springfield, Koenig, DeMaster, & Levitt, 2001). H_2S is proposed to be an energy source for epithelial and other lamina propria cells (Goubern et al., 2007). It has also been reported that H_2S promotes mucus production in the colon (Tomasova, Konopelski, & Ufnal, 2016), and inhibition of H_2S led to inflammation and mucosal injury in the small and large intestines of rats (Wallace et al., 2018).

1.1 Diet Studies: Protein and Fiber Interactive Effects

Numerous studies have been conducted on the effect of dietary fiber, as well as the effect of dietary fiber and fat intake together, on gut microbiome health and disease. In comparison, there is little on protein intake levels, let alone protein and dietary fiber levels combined, even though each significantly affects gut metabolite production and subsequent health outcomes (Holmes et al., 2017). Furthermore, comparisons between controlled studies on protein intake levels are confounded by differences in approach and small sample sizes.

Protein diet studies are often focused primarily on meat consumption and the use of dietary fiber to modulate its effect. In an early study, the effect of a high-protein, low-protein, or wheat bran supplemented high-protein diet on fecal concentrations of SCFAs and ammonia was investigated in healthy men (n = 4) (Cummings et al., 1979). SCFA concentrations showed no changes between the diets, but doubling the amount of meat protein doubled ammonia concentrations, which was not altered by added wheat bran. Adding a resistant starch supplement to a high meat diet in healthy men (n = 8) reduced fecal pH but had no significant effect on gut transit time or fecal concentrations of ammonia or N-nitroso compounds, implying that resistant starch did not significantly alter the occurrence of protein fermentation (Katherine R. Silvester et al., 1997). Similarly, there was no significant difference in BCFA concentrations nor colonic DNA damage between rats on a red meat diet supplemented with either cellulose, potato fiber, or potato resistant starch, although potato fiber increased colonic acetate and butyrate (Paturi et al., 2012).

High-protein low-carbohydrate diets are popular for weight loss, yet very few studies have been conducted on the impact these diets may have on the microbiome. A study of 17 obese men found that regardless of a medium or low carbohydrate intake level a diet high in protein had more markers of protein fermentation compared to the control diet (Russell et al., 2011). However, it should be noted that the control diet had dietary fiber at the recommended dietary level, which is well above the average in populations consuming a standard Western diet. Unfortunately, none of the test diets had a comparably high level of dietary fiber with high protein. An earlier similar study of obese men (n = 19) found that, compared to the control diet, a high-protein low-carbohydrate diet resulted in significantly lower BCFAs, ammonia, and butyrate (Duncan et al.,

2007). As with the Russell et al. (2011) study, the control diet had significantly more dietary fiber and none of the test diets contained a similar level of dietary fiber along with a high protein level.

Most studies looking at the effect of protein on the microbiome only looked at specific sources of protein and used a moderate level of protein or they examined high-protein low-dietary fiber diets without a comparable high-protein high-dietary fiber diet. Based on the popularity of high-protein diets and the generally high protein intake of populations on a Western diet, it is clear that research is needed to determine the impact protein with differing amounts of dietary fiber might have on the microbiome.

Table 2.1. Luminal metabolites which can be formed from amino acid precursors in the large intestine (Adapted from: Blachier et al., 2007)

Amino acid precursors	**Metabolites formed**
Alanine	acetate, ethylamine, propionate
Arginine	agmatine, nitric oxide, putrescine
Aspartate	acetate, succinate
Cysteine	sulfides
Glutamate	acetate, butyrate
Glycine	acetate, methylamine
Histidine	histamine
Isoleucine	2-methylbutyrate
Leucine	isovalerate
Lysine	acetate, butyrate, cadaverine
Methionine	sulfides
Phenylalanine	phenylacetate, phenylethylamine, phenyllactate, phenylpropionate, phenylpyruvate
Threonine	acetate, propionate
Tryptophane	indole, indoleacetate, indolepropionate, 3-methylindole, tryptamine
Tyrosine	4-ethylphenol, hydroxyphenylacetate, hydroxyphenyllactate, hydroxyphenylpropionate, hydroxyphenylpyruvate, p-cresol, phenol, tyramine
Valine	isobutyrate, 2-methylbutylamine
Deamination of amino acids	ammonium
Deamination and fermentation of amino acids	H_2, CO_2, CH_4, lactate, succinate, formate, oxaloacetate

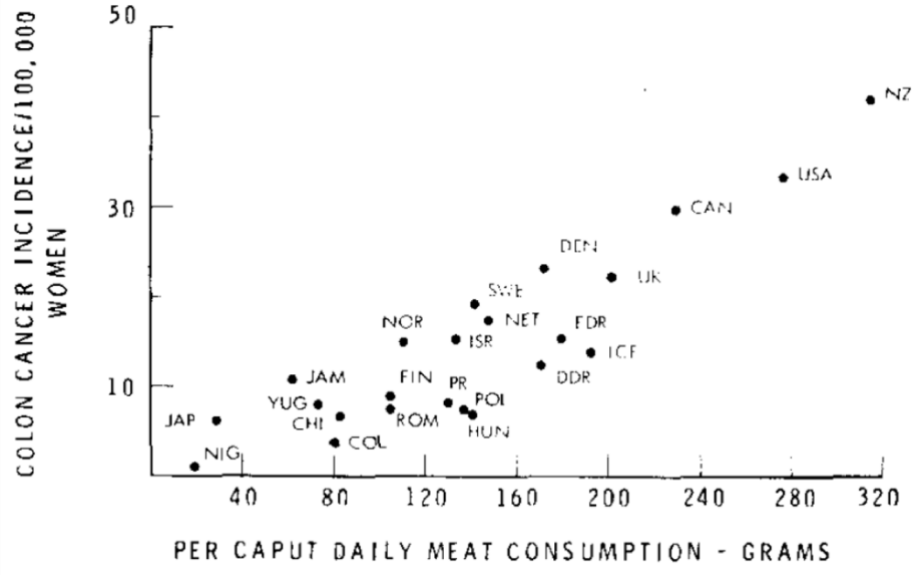

Figure 2.1. "Correlation between incidence of colon cancer in women and *per caput* daily meat consumption in 23 countries (Armstrong & Doll, 1975)."

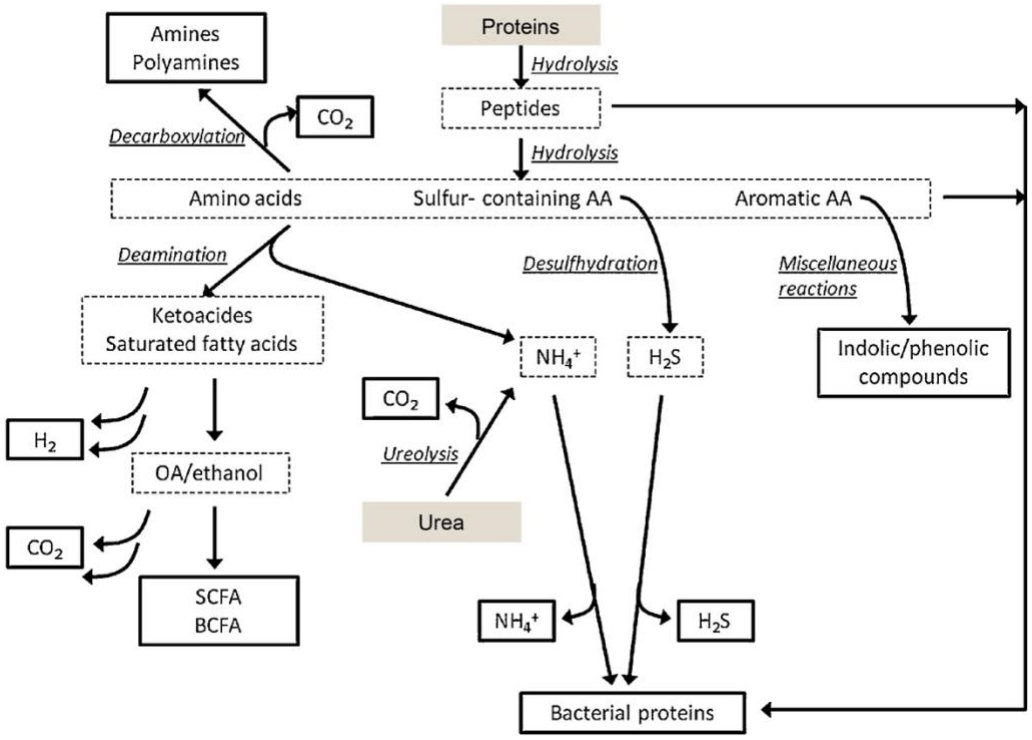

Figure 2.2. Outline of pathways of protein metabolism by gut microbiota, tan-filled box: substrate; dashed-line gray border: intermediary metabolite; solid black border: end product. (From: Davila et al., 2013)

CHAPTER 3. METHODS

3.1 Introduction

In the late 1980's and early 1990's several studies using sudden death victims established and confirmed much of the foundational gut microbiome knowledge applicable to our current work on protein fermentation with different dietary fibers. More specifically, that (1) proteolysis occurs in the colon and bacteria were primarily responsible for its occurrence (Macfarlane et al., 1986), (2) bacterial fermentation of carbohydrates and proteinaceous sources occurs throughout the colon (Cummings et al., 1987), (3) protein fermentation occurs in more distal regions while fermentation of carbohydrates occurs more so in proximal regions (Macfarlane, Gibson, Beatty, et al., 1992), and (4) protein fermentation was responsible for a much larger percentage of SCFA production in the distal colon than the proximal (Macfarlane, Gibson, Beatty, et al., 1992). In combination, these studies largely translated to an understanding that bacterial protein fermentation occurs in more distal regions of the large intestine due to microbial preference for carbohydrates over protein as an energy source. Since colonic diseases occur more often in the distal colon, research turned to scrutinizing physiological consequences of protein fermentation in the human colon.

For decades Western diets have consistently included plentiful protein and chronically low amounts of dietary fiber. Based on estimates, a near equal amount of protein and dietary fiber reach the large intestine on the standard Western diet. Additionally, low-carbohydrate high-protein diets for weight loss have maintained a steady popularity. Since the amount of protein reaching the colon is most strongly tied to the amount of protein consumed (K.R. Silvester & Cummings, 1995), high-protein low-carbohydrate diets are estimated to lead to more fermentable protein than fiber entering the gut. Due to its association with colorectal cancer, research has primarily focused on decreasing gut microbial protein fermentation and increasing beneficial dietary fiber fermentation, while comparatively little attention has been aimed at dietary protein-fiber interaction effects on metabolite production nor how changing proportions of protein and fiber may affect metabolite profiles.

For this study, our goals were two-fold. Firstly, to determine how a changing substrate ratio of protein to fiber affected the type and quantity of metabolites produced. Secondly, we hypothesized how each fiber's specific fermentation characteristics would deter fermentation of protein and ultimately suppress BCFA and ammonia production. Further, it was speculated that, in a protein-fiber substrate mixture, fermentation of the dietary fiber component would produce high concentrations of beneficial SCFAs to counterbalance the less desirable metabolites of protein fermentation.

3.2 Materials and Methods

The experimental design consisted of 4 fiber sources at 3 inclusion levels plus controls (a 100% protein control and a 100% fiber control for each fiber source) and a blank at 2 time points, all in triplicate. The 100% protein control contained protein only substrate. Each fiber source had its own 100% fiber control containing a fiber only substrate. The blank did not contain any substrate and received only the fecal inoculum.

3.2.1 Protein Source and Protein Substrate Preparation

An animal-based polypeptone [Gibco Polypeptone Peptone, Product Code (PC): B11910] consisting of equal parts pancreatic digest of casein (source: bovine) and peptic digest of animal tissue (sources: bovine, equine, porcine) was purchased from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA). Soy protein acid hydrolysate (PC: S1674) was purchased from MilliporeSigma (MilliporeSigma, St. Louis, MO). 85 grams of polypeptone and 15 grams of soy protein hydrolysate were combined to create the protein portion of the protein-fiber substrate mixture for *in vitro* fermentation.

3.2.2 Dietary Fiber Sources and Dietary Fiber Substrate Preparation

Short-chain fructooligosaccharide (FOS) (PC: 111001) was obtained from Ingredion (Ingredion Incorporated, Bridgewater, NJ). Pectin extracted from apple (PC: Classic AF 710, degree of esterification: 33%, galacturonic acid content: 83%) was generously donated by Herbstreith & Fox (Herbstreith & Fox KG, Neuenbürg/Württ., Germany). Wheat bran was gifted from the Mennel Milling Company (Fostoria, OH). An unmodified raw potato starch was used as a source of type

2 resistant starch (RS2) ("Premium Quality Unmodified Potato Starch;" Bob's Red Mill Natural Foods, Inc., Milwaukie, Oregon). With the exception of FOS, all fiber substrates for *in vitro* fermentation underwent *in vitro* upper gastrointestinal (GI) digestion (as described below in detail). FOS would not have survived the dialysis portion of the *in vitro* upper GI digestion procedure due to its small molecular weight and was therefore excluded. The pure FOS used for this study is known to be indigestible in the upper GI tract of humans.

3.2.2.1 De-fatting of Wheat Bran; WB+PS Preparation

To prepare the WB+PS sample prior to *in vitro* upper GI digestion, wheat bran was first defatted by twice suspending it with hexane (bran : hexane 1:7, w/v) for 60 minutes with constant stirring, after which hexane was removed via filtration. The wheat bran was then allowed to air-dry overnight. After drying, wheat bran was sieved to a size range of 300-500 µm using a sieving machine (Portable Sieve Shaker Model RX-24, sieving machine and screens both from W.S. Tyler Combustion Engineering, Inc., Mentor, OH). Equal weights of defatted wheat bran and raw potato starch were used to make the WB+PS samples which were then subjected to *in vitro* upper GI digestion (detailed below).

3.2.2.2 Even Mixture Sample Preparation

Pectin, wheat bran, and potato starch were combined and underwent *in vitro* upper GI digestion (described below), and then FOS was added. The final composition of the Even Mixture (by weight) for *in vitro* fermentation was 1/3 FOS, 1/3 Pectin, 1/6 wheat bran, and 1/6 potato starch.

3.2.2.3 *In vitro* Upper GI Digestion Simulation

The *in vitro* upper GI digestion procedure (Mishra & Monro, 2009; Yang, Keshavarzian, & Rose, 2013) simulates the passage and digestion of starch and proteins through the upper GI tract (from stomach through small intestine) of humans. Fiber substrates (pectin, WB+PS, and the even mixture before FOS addition) were subjected to this *in vitro* upper gastrointestinal digestion with slight modification to prevent cooking/gelatinization of the raw potato starch. Briefly, the fiber

substrates were suspended in 37°C distilled water[5] and 1M HCl was used to decrease the pH to 2.5. The fibers were enzymatically treated with 100 mg·mL^{-1} of pepsin dissolved in 50 mM HCl (≥250 units/mg, P-7000, MilliporeSigma, St. Louis, MO) for 30 minutes at 37°C with constant stirring (700 rpm). Sodium maleate buffer (0.1M, pH 6) containing 1mM $CaCl_2$ was added followed by pH adjustment to 6.9 ± 0.1 using 1M $NaHCO_3$. Then, pancreatin (125mg/mL, P-7545, MilliporeSigma, St. Louis, MO) and amyloglucosidase (3260 units/mL, E-AMGDF, Megazyme International, Wicklow, Ireland) were added and the mixture incubated at 37°C for 6 hours with constant stirring (700 rpm). Finally, 6-8 kDa membrane tubing (PC: 132675, Repligen Corporation, Waltham, MA) was used to dialyze the fibers against distilled water for 36 hours with water changes every 6-12 hours, then freeze dried.

3.2.3 *In vitro* Fecal Fermentation

In vitro fermentation was carried out as previously described (Lebet, Arrigoni, & Amadò, 1998) under an 85% N_2, 5% CO_2, and 10% H_2 atmosphere in an anaerobic chamber. Carbonate-phosphate buffer[6] (Durand, Dumay, Beaumatin, & Morel, 1988) was prepared and sterilized by autoclaving at 121°C for 20 minutes. After cooling to room temperature, oxygen was removed from the buffer by bubbling with carbon dioxide; cysteine hydrochloride (0.25 g/L of buffer) was added as a reducing agent. The buffer was then immediately placed into an anaerobic chamber for 24 hours before using.

Glass test tubes were sterilized by autoclaving at 121°C for 40 minutes. Prepared protein and dietary fibers (FOS, Pectin, WB+PS, or Even Mixture) were then weighed into each tube so that each contained 50 ± 0.3 mg substrate (except the blank containing 0 mg substrate). Substrates were prepared in triplicate for each time point (24 and 48 hours for controls and protein-fiber mixtures, 0, 24, and 48 hours for the blanks) with protein and dietary fiber amounts shown in Table 3.1 below, then placed into the anaerobic chamber overnight. The naming convention of substrates

[5] To avoid excessive viscosity, pectin was hydrated in excess ice cold distilled water with vigorous stirring, slowly heated with continued stirring until all clumps were completely dissolved, then cooled to 37°C to begin the procedure. All enzymatic treatments were adjusted accordingly to account for the excess dilution.

[6] The carbonate phosphate buffer contained urea (6.6 mM) and ammonium (13.7 µM) with a total nitrogen concentration of 16.4 mM.

was based on the fiber source and percent fiber of the total substrate. An example is included in Table 3.1.

Table 3.1. Weights of protein and dietary fibers used for protein-fiber substrate mixtures, percent fiber inclusion, and example naming structure

Prepared Protein Substrate (mg)	Prepared Dietary Fiber Substrate (mg)	Total Weight of Substrate (mg)	Fiber Inclusion (of total substrate)	Example Naming Convention using FOS
0.00	0.00	0.00	0%	Blank
50.00 ± 0.13	0.00	50.00	0%	100% protein control
37.50 ± 0.19	12.50 ±0.19	50.00	25%	FOS (25)
25.00 ± 0.18	25.00 ± 0.18	50.00	50%	FOS (50)
12.50 ± 0.17	37.50 ± 0.17	50.00	75%	FOS (75)
0.00	50.00 ± 0.16	50.00	100%	100% FOS fiber control

The following day, 4 mL of carbonate-phosphate buffer was added to each tube. Fecal samples were provided by 2 healthy donors (1 female and 1 male) who were consuming their routine diets and who had not taken antibiotics within the previous 6-month period. Fecal samples were kept on ice in tightly sealed plastic tubes, immediately transferred into the anaerobic chamber, and used within 3 hours of collection. Fecal slurry was prepared by first homogenizing fecal samples and the previously prepared anaerobic carbonate-phosphate buffer [feces:buffer 1:3 (w/v)] followed by filtration through 4 layers of cheesecloth. Each test tube was then inoculated with 1 mL fecal slurry, closed with a sterilized butyl rubber stopper (Chemglass Life Sciences), sealed with an aluminum seal (Chemglass Life Sciences), and placed in a 37°C shaking incubator.

3.2.4 Total Gas Production, pH Measurement, and SCFA and Ammonia Sample Collection

At each time point, tubes were removed from the incubator, gas and pH measurements were recorded, and samples were taken for later quantification of SCFAs (including BCFAs) and ammonia. Total gas production was measured by passage of a needle attached to a graduated syringe through the rubber stopper and recording the volume displaced by the plunger. The seals and stoppers were then removed. Upon removal of the stopper, two separate 1 mL aliquots were

immediately collected from each tube, one for SCFA analysis and one for ammonia analysis, then stored at -80°C until further analysis. The remaining fermentation sample was then transferred to a 15 mL plastic tube for pH measurement.

3.2.5 SCFA and BCFA Analysis

SCFA analysis was carried out as previously described (Tuncil et al., 2017) with slight modification. An internal standard mixture was prepared by combining 157.5 µL of 4-methylvaleric acid, 1.47 mL of 85% phosphoric acid, and 39 mg $CuSO_4$ pentahydrate, then bringing the final volume to 25 mL with purified water. External standards were prepared by serial diluting a prepared standard solution [0.6M acetic acid (500 µL), 0.15M propionic acid (500 µL), 0.15M butyric acid (500 µL), 0.15M iso-butyric acid (250 µL), and 0.15M iso-valeric acid (250 µL)]. Samples for SCFA and BCFA analysis were allowed to defrost at room temperature and centrifuged at 15,115 x g for 10 minutes. Supernatants (0.4 mL) and external standards (0.4 mL) were transferred to separate GC vials (DWK Life Sciences), then 100 µL of internal standard was added to each. Samples (4 µL) were injected into a gas chromatograph equipped with a fused silica capillary column (NukonTM, Supelco No: 40369-03A, Bellefonte, PA) and a flame ionization detector (GC-FID 7890A, Agilent Technologies, Inc., Santa Clara, CA) with the following conditions: injector temperature at 230 °C; initial oven temperature at 100 °C; temperature increase of 8 °C·min^{-1} to 200 °C with a hold for 3 minutes at final temperature. Helium was used as a carrier gas at 0.75 mL·min^{-1}. Quantification was carried out by measuring the peak areas for acetate, propionate, butyrate, iso-butyrate, and iso-valerate relative to 4-methylvaleric acid.

3.2.5.1 Normalization of BCFA Data

BCFAs are formed exclusively from proteinaceous substrate, specifically branched-chain amino acids. This distinction allowed for the data to be normalized (Equation 1) in order to conduct comparisons between all samples (containing protein) despite unequal initial quantities of protein substrate.

Equation 1.

$$\frac{Detected\ mM\ of\ BCFA}{grams\ of\ protein\ added\ as\ substrate\ at\ time\ 0} = BCFA\ (mM/g\ protein\ basis)$$

3.2.6 Ammonia

Ammonia was measured using the Megazyme Rapid Ammonia Assay Kit (PC: K-AMIAR; Megazyme International, Wicklow, Ireland) in microplate format. Briefly, it is a spectrophotometric method using glutamate dehydrogenase (GIDH) to enzymatically convert 2-oxoglutarate, reduced nicotinamide-adenine dinucleotide phosphate (NADPH), and ammonium ions into L-glutamic acid, $NADP^+$, and water. NADPH consumption is measured by a decrease in absorbance at 340 nm; the amount of $NADP^+$ is stoichiometric with the amount of ammonia (*Ammonia Rapid Assay Procedure K-AMIAR*, 2018). Samples for ammonia analysis were defrosted at room temperature and centrifuged at 1,500 x g for 10 minutes. Supernatants (100 µL) were removed to new tubes to be deproteinized before analysis. Samples were deproteinized by adding 100 µL of ice-cold 1M perchloric acid (PC: A2296, Thermo Fisher Scientific) with mixing and centrifuged at 1,500 x g for 10 minutes. Finally, 100 µL of the resulting supernatant was neutralized with 50 µL 1M potassium hydroxide. Ammonia assay was performed by first pipetting distilled water into a 96 well plate, followed by the deproteinized sample, then the provided buffer (containing 2-oxoglutarate), and NADPH. After 2 minutes of mixing, absorbance (A_1) at 340 nm was read (SpectraMax 190, Molecular Devices Corporation, Sunnyvale, CA). Then GIDH was added to begin the reaction, and after 5 minutes of mixing, the second absorbance (A_2) was recorded. A blank and a three-point calibration curve was performed concurrently. Ammonia (mg/mL) was calculated using the calibration curve linear equation (R_2=0.9997) and the change in absorbance of the samples, then multiplying by the dilution factors introduced from deproteinization (df=3) and dilution of sample to fit the assay range (df=2). Ammonia was then converted to and reported on a millimolar basis.

3.2.7 Statistical Analysis

All statistical analyses were performed by ANOVA using JMP® (version 13.2 SAS Institute Inc., Cary, NC, 1989-2019). When ANOVA was significant, Tukey's honestly significant difference (HSD) post-hoc test was conducted to differentiate group means. All tests were conducted at the

$\alpha = 0.05$ level. Blanks containing inoculum, but no substrate, were not included in statistical analysis unless otherwise noted.

CHAPTER 4. RESULTS

4.1 Gas Production

Gas production results are presented in Figure 4.1 and Figure 4.2. There was a significant interaction effect of fiber source and fiber inclusion on gas response [$F(4, 12) = 25.13$, $p = <.0001$]. Correlation analysis indicated a significant positive correlation of gas production with proportion of fiber included in the protein-fiber substrate ($r = 0.8805$, $p < 0.0001$).

Gas production increased as fiber inclusion level increased as shown in Figure 4.1. Markedly less gas was produced by the 100% protein control ($\overline{x} = 4.6 \pm 0.5$ cm^3, n=6) compared to that of the 100% fiber controls ($\overline{x} = 11.4 \pm 1.7$ cm^3, n=24). All protein-fiber mixture samples had significantly higher gas production compared to the 100% protein control.

4.2 pH

Results for pH are shown in Figure 4.1 and Figure 4.3. There was a significant interaction effect of fiber source and fiber inclusion on pH response [$F(4, 12) = 29.78$, $p = <.0001$].

The pH range was 6.6 – 8.4. There was an inverse relationship between pH and fiber inclusion level. Each fiber type showed decreasing pH with increasing fiber. Compared to the 100% protein control, all samples except WB+PS (25) had lower pH ($p < 0.05$). FOS had significantly lower pH than the 100% protein control and all other tested fibers at each inclusion level and time point (Figure 4.3).

4.3 Branched-Chain Fatty Acids

BCFA results were normalized based on the quantity of protein substrate added at time zero as shown in Equation 1, and presented as millimoles BCFAs per gram of initial protein substrate (mM/g protein basis). The data prior to normalization (as total millimolar BCFAs) are found in the Appendix (Table A.1).

4.3.1 Total BCFAs

There was a significant interaction effect of time, fiber source, and fiber inclusion level on cumulative total BCFA (mM/g protein basis) ($p < 0.0001$). BCFA (mM/g protein basis) results are shown in Figure 4.4 and Figure 4.7 and statistical test results presented in Table 4.1.

FOS and Even Mix were the only fiber sources to significantly inhibit BCFA production during fermentation. In the first 24 hours of fermentation, only FOS (75) and Even Mix (75) had significantly less total BCFAs (mM/g protein basis) than the 100% protein control. FOS is a fast-fermenting fiber that was likely consumed completely within the first 6 hours of fermentation. Yet, FOS had a continued suppression effect of BCFAs (mM/g protein basis) throughout the 48-hour fermentation period, likely due to significantly lower pH compared to other protein-fiber mixture samples. Interestingly, total BCFAs (mM/ g protein basis) after 48 hours were significantly lower than the 24-hour response for FOS (75), a result that did not occur in other protein-fiber mixture samples. The most likely explanation is microbial utilization of BCFAs for growth in the second 24-hour period.

Unexpectedly, 24-hour WB+PS (25) had a significantly higher total BCFA concentration (mM/g protein basis) than the 100% protein control, suggesting an increased rate of protein fermentation at 24 hours since the 48 hour values were similar to that of the 100% protein control.

4.3.2 Iso-Butyrate

There was a significant interaction effect of fiber source, fiber inclusion, and time on cumulative iso-butyrate production (mM/g protein basis) ($p < 0.0001$). Results are presented in Figure 4.5 and Figure 4.7 and statistical test results presented in Table 4.1.

Compared to the 100% protein control at 24 hours, WB+PS (25) had significantly higher concentrations of iso-butyrate (mM/g protein basis) while Even Mix (75) had significantly less. After 48 hours, compared to the 100% protein control, only FOS (50) and FOS (75) had significantly less iso-butyrate production.

In the first 24 hours, FOS and Pectin had a similarly steady iso-butyrate response that did not appear to be influenced by inclusion level. After 48 hours fermentation, FOS differentiated itself from Pectin by having a declining iso-butyrate response as fiber inclusion level increased while Pectin remained relatively unchanged. The inhibitory effect Even Mix (75) showed at 24 hours did not continue through the 48-hour period. Compared to the 100% protein control, WB+PS (75) produced significantly more iso-butyrate at 24 hours, but by 48 hours there was no statistical difference, which indicates WB+PS (75) increased the rate of iso-butyrate production. Overall, fiber inclusion had a minimal effect on iso-butyrate production regardless of fiber proportion in the protein-fiber mixture.

4.3.3 Iso-Valerate

There was a significant interaction effect of fiber source, fiber inclusion, and time on cumulative iso-valerate (mM/g protein basis) response ($p < 0.0001$). Results are presented in Figure 4.6 and Figure 4.7 and statistical test results presented in Table 4.1.

There were higher concentrations of iso-valerate than iso-butyrate regardless of fiber inclusion. However, both iso-valerate and iso-butyrate had similar concentration patterns based on fiber source, inclusion level, and fermentation time. At 24 hours, there was a statistical difference between the 100% protein control and three samples. Similar to iso-butyrate responses, WB+PS (25) had a significantly higher concentration of iso-valerate compared to the 100% protein control (130.6 mM/g protein and 98.9 mM/g protein respectively). In contrast, Even Mix (75) and FOS (75) had significantly lower values (55.9 mM/g protein and 51.4 mM/g protein). Overall, the WB+PS samples (25, 50, and 75% inclusion) had the highest iso-valerate concentrations in the first 24 hours. After 48 hours, WB+PS samples had nearly identical iso-valerate concentrations compared to the 100% protein control (Figure 4.6), and interestingly, any effect of inclusion level disappeared, again indicating that the rate of formation was likely sped up in the first 24 hours but cumulative totals were relatively steady. At 48 hours, four samples, FOS (50 and 75), Pectin (75), and Even Mixture (75), had significantly less iso-valerate responses than the 100% protein control. The 48-hour FOS (75) had the lowest overall level with an iso-valerate response of 20.0 mM/g, which was significantly lower than all other samples at either time point and less than the 100%

protein control by nearly 5-fold. It was also significantly lower than its respective 24-hour response, indicating occurrence of microbial utilization between 24 and 48 hours fermentation.

With the exception of protein-WB+PS mixtures, increased inclusion of fiber decreased iso-valerate, although generally without significance. At 48 hours, all levels of WB+PS inclusion were nearly identical to one another and to the 100% protein control. The value of the 100% protein control at 48 hours was 110.2 mM/g, whereas the values for WB+PS at 25, 50, and 75% inclusion were 108.1 mM/g, 108.7 mM/g, and 110.3 mM/g, respectively.

4.4 Ammonia

Ammonia results are shown in Figure 4.8. Even though ammonia is primarily a product of protein fermentation, there was considerable variability in amount among the 100% fiber controls. Since there was measurably more ammonia in the blanks than some of the 100% fiber controls, the blanks were included in statistical analysis and are presented in the figures. There was a significant interaction effect of fiber source and fiber inclusion on ammonia response [$F(4, 12) = 39.12$, $p < 0.0001$].

Measurable quantities of ammonia were detected in the 100% fiber controls and blanks, including the blank at the start of the experiment. The fermentation buffer contained 13.7 µM of ammonium which does not account for the 9.7 ± 0.9 mM present at time zero in the blank. Ammonia was most likely within the fecal material used for the inoculum or there may have been contamination during fecal collection by contact with urine. In the blank, ammonia increased from 9.4 ± 0.7 mM at 24 hours to 12.2 ± 0.6 mM at 48 hours fermentation. This may be partially explained by microbial urea hydrolysis since the fermentation buffer contained 6.6 mM urea. However, this does not account fully for the ammonia measured and therefore indicates the occurrence of protein fermentation. The most likely explanation is the presence of proteinaceous material within the fecal material used for inoculum. However, total BCFAs were below detectable limits at each time point for the blank and 100% fiber controls which indicates there was no substantial occurrence of protein fermentation, or at the very least, branched chain amino acids were not substantially fermented.

The 100% protein control had the highest level of ammonia, but it was not significantly different from any protein-fiber mixture samples at 25% fiber inclusion. The only fiber to differ significantly from the 100% protein control at 50% inclusion was FOS (50), although as can be seen in Figure 4.8, the decrease was relatively small compared to the steep decline for all protein-fiber mixture samples at 75% inclusion. Also of note, the blanks containing inoculum, but no substrate, had a measurable concentration of ammonia that was significantly higher than FOS, WB+PS, and 100% Even Mix fiber controls. There were some significant differences within the 100% fiber controls which may be indicative of differing levels of microbial utilization of ammonia as a nitrogen source. The 100% FOS fiber control had the lowest ammonia production compared to all other samples including the blank.

Significant suppression of ammonia required at least 50% of FOS inclusion or 75% inclusion of the other tested fibers. It was more than likely the reduction of protein and not the presence of fiber that had the biggest influence on reducing ammonia levels since significant decreases in ammonia concentration generally required protein to be reduced by 75%.

Overall, incrementally lowering protein and increasing fiber in the fermentation substrate did not greatly affect ammonia response until fiber comprised over half the substrate. Among the fibers tested, FOS had the greatest effect on lowering ammonia. Pectin and WB+PS had a lesser effect on lowering ammonia with the Even Mixture being marginally more effective.

4.5 Short-Chain Fatty Acids

In addition to the results and figures presented below, a complete table of SCFA data (acetate, propionate, butyrate, and total) for all samples, including the blank, is located in the appendix (Table A.2).

4.5.1 Total SCFAs

Total SCFAs are the sum of acetate, propionate, and butyrate; results are shown in Figure 4.9 and Figure 4.13 and statistical effect test results in Table 4.2. There was a significant interaction effect

of fiber source, fiber inclusion level, and fermentation time on total cumulative SCFA response [$F(1, 17) = 10.83$, $p < 0.0001$].

The cumulative total SCFA response for all samples except the blank ranged from 33.7 mM - 72.7 mM (blank range = 0.5 – 4.5 mM). Differentiated by time, the range was 33.7 – 57.4 mM at 24 hours and 43.2 – 72.7 mM at the 48-hour time point.

As shown in Figure 4.13, substrates containing FOS, Pectin, and Even Mixture had similar total SCFA responses in the first 24 hours regardless of inclusion amount, but increasing fiber inclusion showed an upward trend in total SCFA response. Conversely, during this same period, there was a linear downward trend for WB+PS in which no significant difference was found between the 100% protein control and WB+PS (50), WB+PS (75), or the 100% WB+PS fiber control. Only WB+PS (25) produced significantly more total SCFAs than the 100% protein control. Surprisingly, at 24 hours there was only a difference of 0.16 mM between the 100% protein control and the 100% fiber control for WB+PS. All levels of Pectin and Even Mix had significantly higher total SCFAs than the 100% protein control after 24 hours.

After 48 hours fermentation, there was greater differentiation between the fiber sources. FOS (50), FOS (75), and the 100% FOS fiber control were significantly higher than all other fibers and the 100% protein control. Protein-fiber mixtures containing WB+PS continued to have the lowest response, though they no longer trended downward with increased WB+PS inclusion.

4.5.2 Acetate

Acetate production results are shown in Figure 4.10 and Figure 4.13 and statistical effect test results in Table 4.2. There was a significant interaction effect of fiber source, fiber inclusion level, and fermentation time on cumulative acetate response [$F(1, 17) = 10.74$, $p < 0.0001$].

Results for acetate production are shown in Figure 4.10. Acetate made up the greatest proportion of SCFAs produced, ranging from 21.4 – 52.4 mM. At both time points, the 100% protein control had the lowest acetate levels.

As shown in Figure 4.13, after 24 hours incubation, FOS, Pectin, and Even Mix protein-fiber mixtures trended upwards with increasing fiber inclusion; in contrast, WB+PS trended downwards. Overall, the general trajectories of acetate production between the fibers at both time points mirrored those of total SCFA production; FOS, Pectin, and the Even Mixture trended upward with increasing inclusion levels, becoming more distinct between the fibers after 48 hours fermentation while WB+PS fiber substrate trended downward in the first 24 hours and then showed a slight upward trend after 48 hours.

While the other fibers at both time points had significantly more acetate starting at the 50% inclusion level and continued upwards, WB+PS samples were not differentiated much from the 100% protein control. At 24 hours, only WB+PS (25) had significantly more acetate than the 100% protein control, and at 48 hours none of the protein-WB+PS mixtures differed from the 100% protein control (although the 100% WB+PS fiber control had significantly more than the 100% protein control).

4.5.3 Propionate

Propionate production results are shown in Figure 4.11 and Figure 4.13 and statistical effect test results in Table 4.2. There was a significant interaction effect of fiber source, fiber inclusion level, and fermentation time on cumulative propionate response [$F(1, 17) = 7.85$, $p < 0.0001$].

As shown in Figure 4.13, protein-fiber mixtures containing FOS were the only samples in which propionate concentration trended upwards with increasing fiber inclusion at either time point. The other tested fibers generally trended downwards. In fact, 100% fiber controls for Pectin, WB+PS, and Even Mix fibers produced significantly less propionate than the 100% protein control.

Other than FOS, increasing fiber inclusion level suppressed propionate response. Only one level of one protein-fiber mixture [WB+PS (25)] had significantly more propionate than the 100% protein control at 24 hours, however even this edge was lost in the second 24-hour fermentation period. After 48 hours, only FOS (75) had significantly more propionate than the 100% protein control, whereas Pectin (75) and Even Mixture (75) both produced significantly less propionate.

4.5.4 Butyrate

Butyrate production results are shown in Figure 4.12 and Figure 4.13 and statistical effect test results in Table 4.2. There was a significant interaction effect of fiber source, fiber inclusion level, and fermentation time on cumulative butyrate response [$F(1, 17) = 7.42$, $p < 0.0001$].

Comparisons between fiber sources show that in the first 24 hours protein-fiber mixtures had surprisingly similar downward trends in butyrate production (Figure 4.13), although within each fiber source differences were mostly indistinct among fiber inclusion levels (Figure 4.12). At each fiber inclusion level, WB+PS and Pectin protein-fiber mixture samples were significantly different from one another in butyrate production; out of all the fibers tested, protein-WB+PS mixtures continually had the highest and protein-Pectin mixture samples the lowest butyrate concentrations within each fiber inclusion level. After 48 hours fermentation, the trend reversed somewhat for FOS and WB+PS protein-fiber mixtures, with each having significantly more butyrate at 75% inclusion compared to its 24-hour counterpart. Compared to the protein-fiber mixtures, 100% fiber controls were much more distinct from one another, especially in the first 24 hours in which each 100% fiber control was significantly different from the other (Figure 4.12). At 48 hours, FOS and WB+PS 100% fiber controls had significantly higher butyrate levels compared to the 100% protein control.

As shown in Figure 4.12, only one protein-fiber mixture at one level, namely WB+PS (25), had higher butyrate production than the 100% protein control, which occurred only in the first 24 hours. After 48 hours, there were no protein-fiber mixtures with higher butyrate than the 100% protein control. There was no evidence of significant differences between the 100% protein control and FOS or Even Mix protein-fiber mixtures at any fiber inclusion level or time point. However, several protein-Pectin mixtures had significantly less butyrate than the 100% protein control, namely Pectin (75) at 24 hours, and Pectin (50) and Pectin (75) at 48 hours. The 100% Pectin fiber control also had the lowest butyrate of all the samples at both time points.

Overall, the results show that inclusion of fiber with a protein substrate made remarkably little difference in production of butyrate. It is possible that the presence of protein may be disadvantageous for generation of butyrate via fiber fermentation.

Table 4.1. BCFA and ammonia effect test p-values after significant ($p < 0.001$) ANOVA. BCFA tests performed on mM/gram protein basis data.

Effect	p-values			
	Total BCFA	Iso-Butyrate	Iso-Valerate	Ammonia
Fiber Source (S)	1.0000	1.0000	1.0000	1.0000
Fiber Inclusion (I)	<.0001*	0.0234*	<.0001*	<.0001*
S x I	<.0001*	<.0001*	<.0001*	<.0001*
Fermentation Time (T)	0.0028*	0.1844	0.0002*	<.0001*
S x T	1.0000	1.0000	1.0000	1.0000
I x T	0.0035*	<.0001*	0.0147*	<.0001*
S x I x T	<.0001*	<.0001*	<.0001*	0.6316

Table 4.2. SCFA effect test p-values after significant ($p < 0.001$) ANOVA.

Effect	p-values			
	Acetate	Propionate	Butyrate	Total SCFAs
Fiber Source (S)	1.0000	1.0000	1.0000	1.0000
Fiber Inclusion (I)	<.0001*	<.0001*	<.0001*	<.0001*
S x I	<.0001*	<.0001*	<.0001*	<.0001*
Fermentation Time (T)	<.0001*	<.0001*	<.0001*	<.0001*
S x T	1.0000	1.0000	1.0000	1.0000
I x T	0.0005*	<.0001*	<.0001*	0.0001*
S x I x T	<.0001*	<.0001*	<.0001*	<.0001*

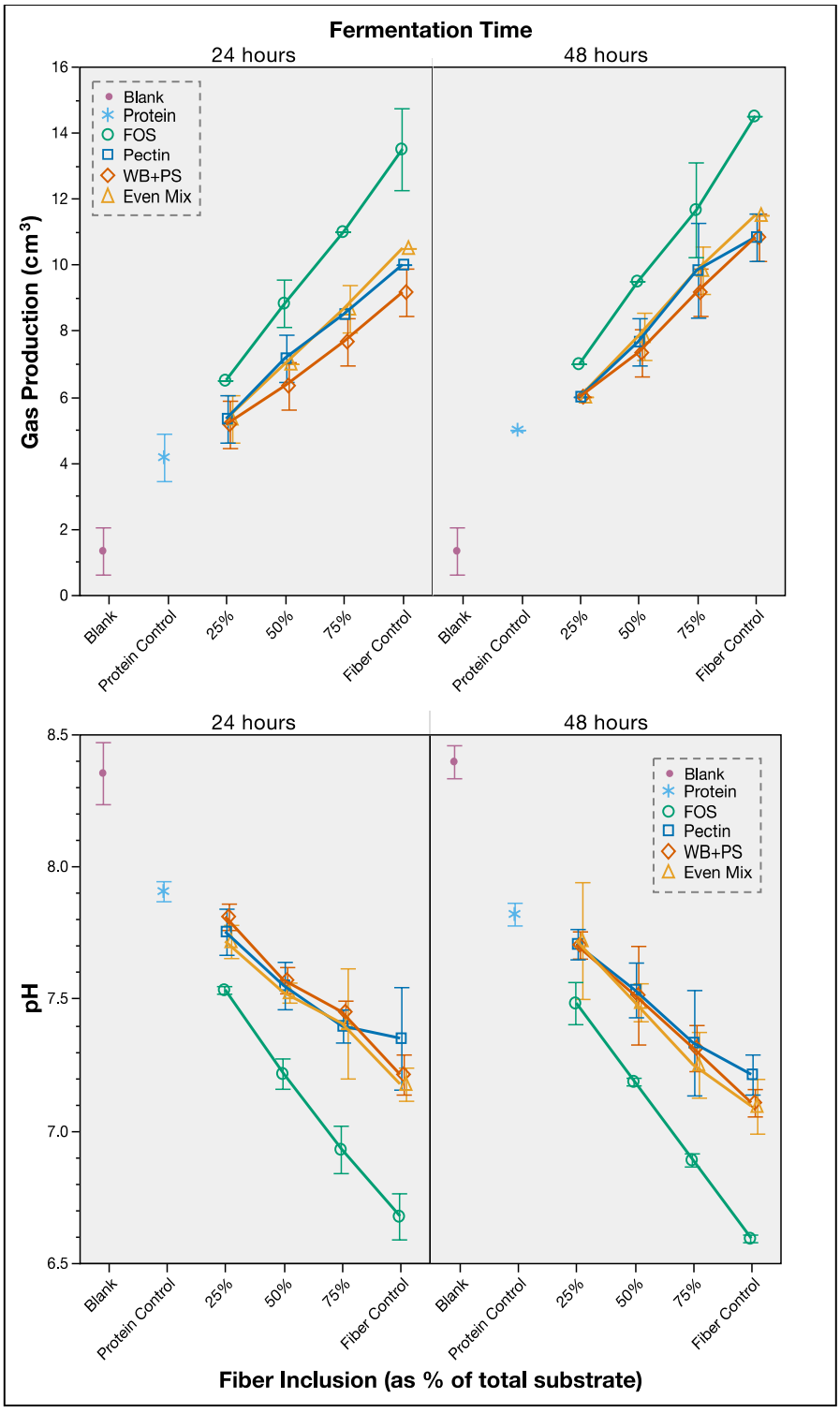

Figure 4.1. Mean gas production (top) and pH (bottom) trends after 24 and 48 hours of fermentation of protein-fiber substrate mixtures (n=3). Error bars = 95% confidence interval. X-axis represents the percentage of fiber in a protein-fiber substrate mixture. 'Blank' = no substrate, inoculum only, 'Protein Control' = 100% protein, 0% fiber substrate, 'Fiber Control' = 100% fiber substrate, 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

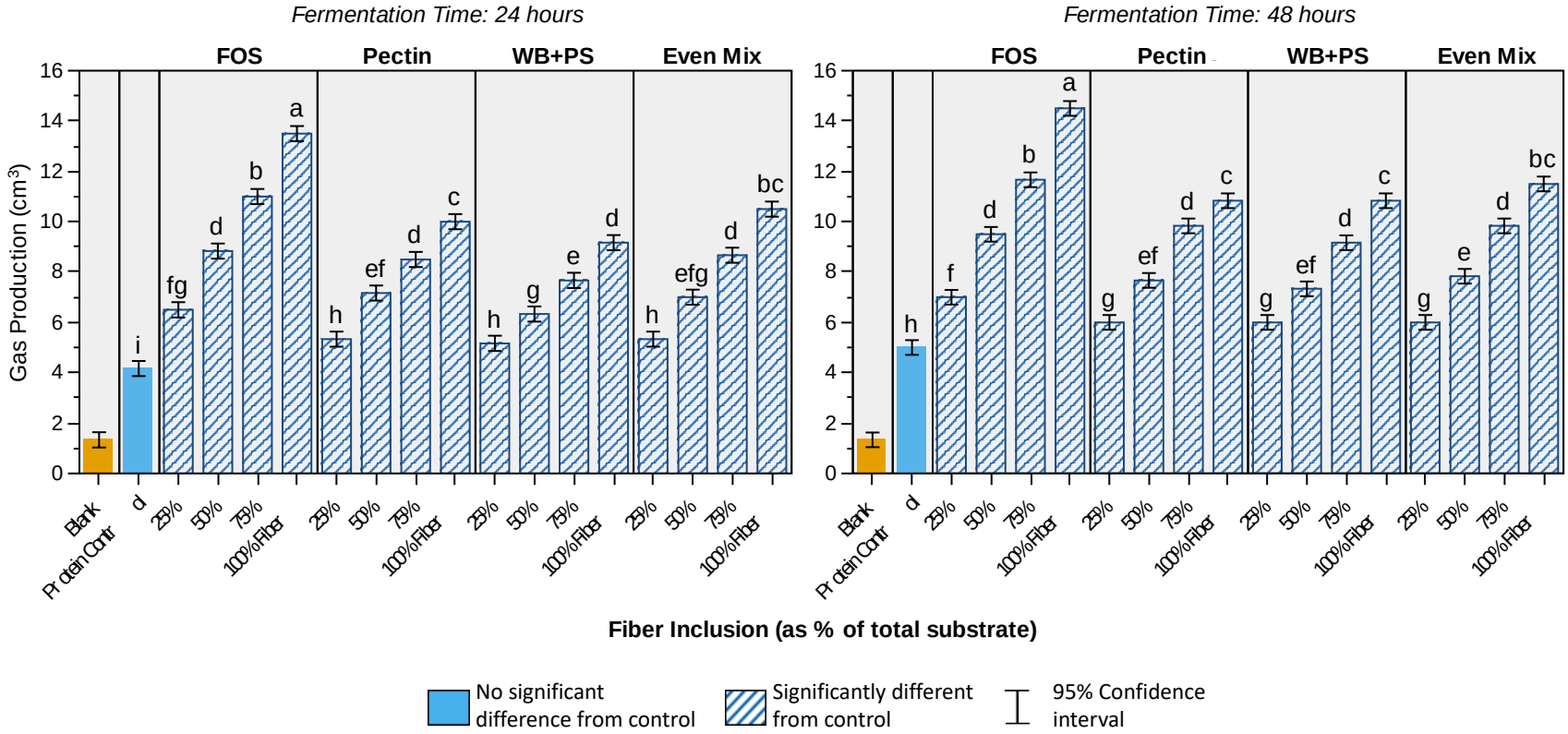

Figure 4.2. Least square mean gas production after 24 and 48 hours fermentation of protein-fiber substrate mixtures (n=3), grouped by fiber substrate source (top). X-axis represents the percentage of fiber in the protein-fiber fermentation substrate mixture. 'Blank' = no substrate, inoculum only, 'Protein Control' = 100% protein, 0% fiber substrate. Within each time point, bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance (the Blank was not included in statistical testing). 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

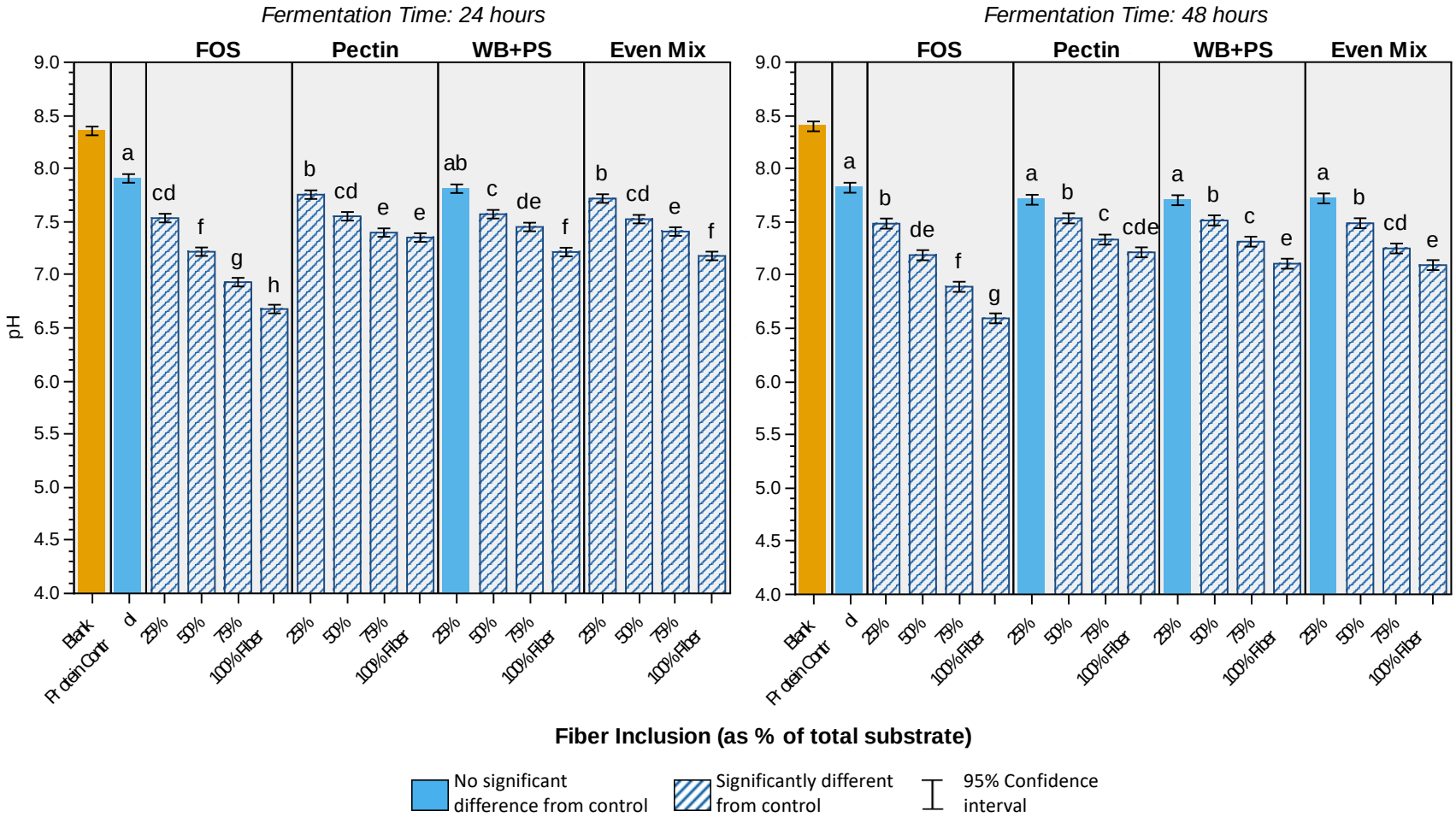

Figure 4.3. Least square mean pH values after 24 and 48 hours fermentation of protein-fiber substrate mixtures (n=3), grouped by fermentation time and fiber substrate source (top). X-axis represents the percentage of fiber in the protein-fiber fermentation substrate mixture. 'Blank' = no substrate, inoculum only, 'Protein Control' = 100% protein, 0% fiber substrate. Within each time point, bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance (the Blank was not included in statistical testing). 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

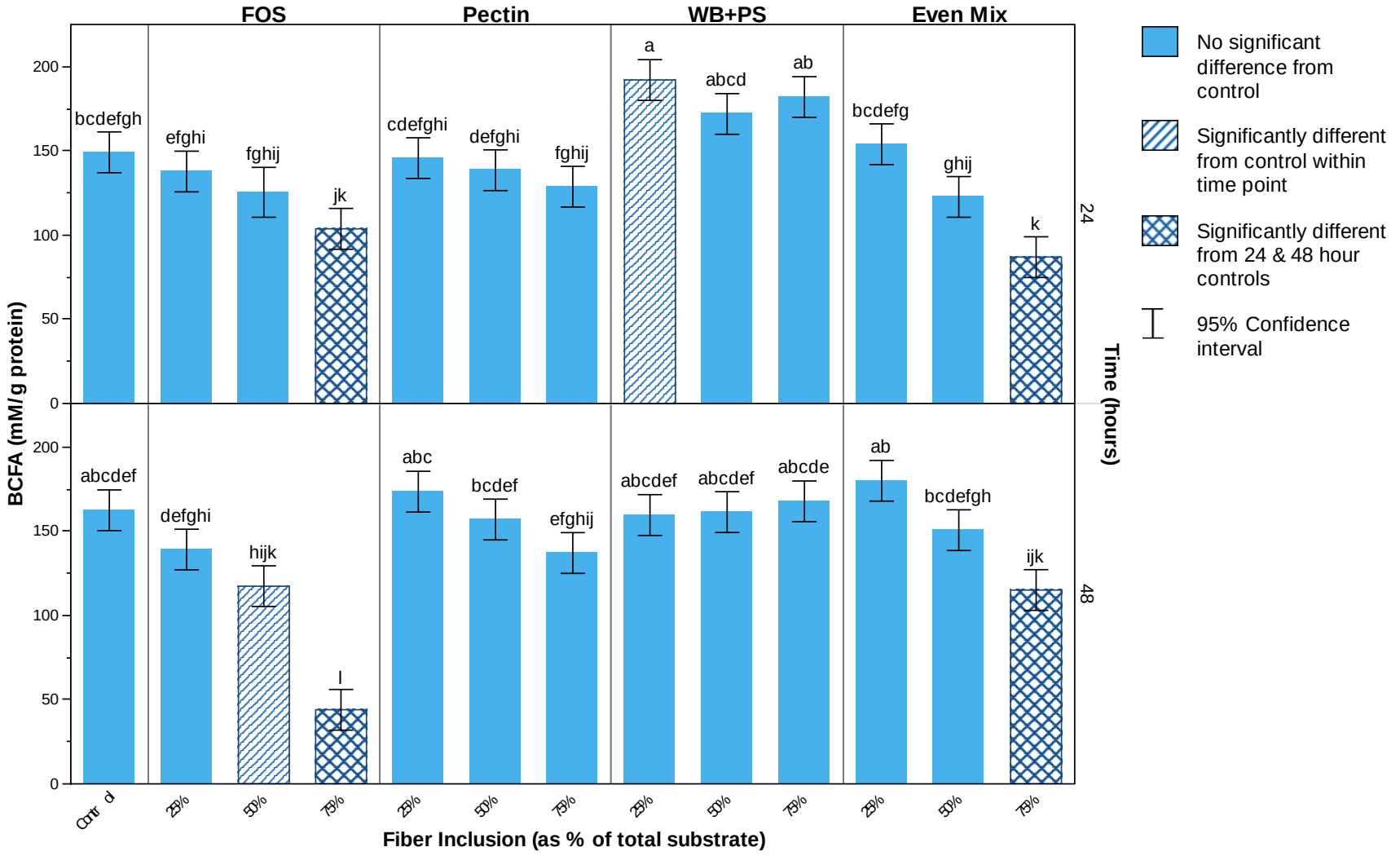

Figure 4.4. Least square mean total BCFA (mM/gram initial protein) after 24 and 48 hours of fermentation (secondary y-axis) of protein-fiber substrate mixtures (n=3), grouped by fiber substrate source (top). X-axis represents the initial proportion of fiber in the substrate mixture. 'Control' = 100% protein, 0% fiber substrate. Bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

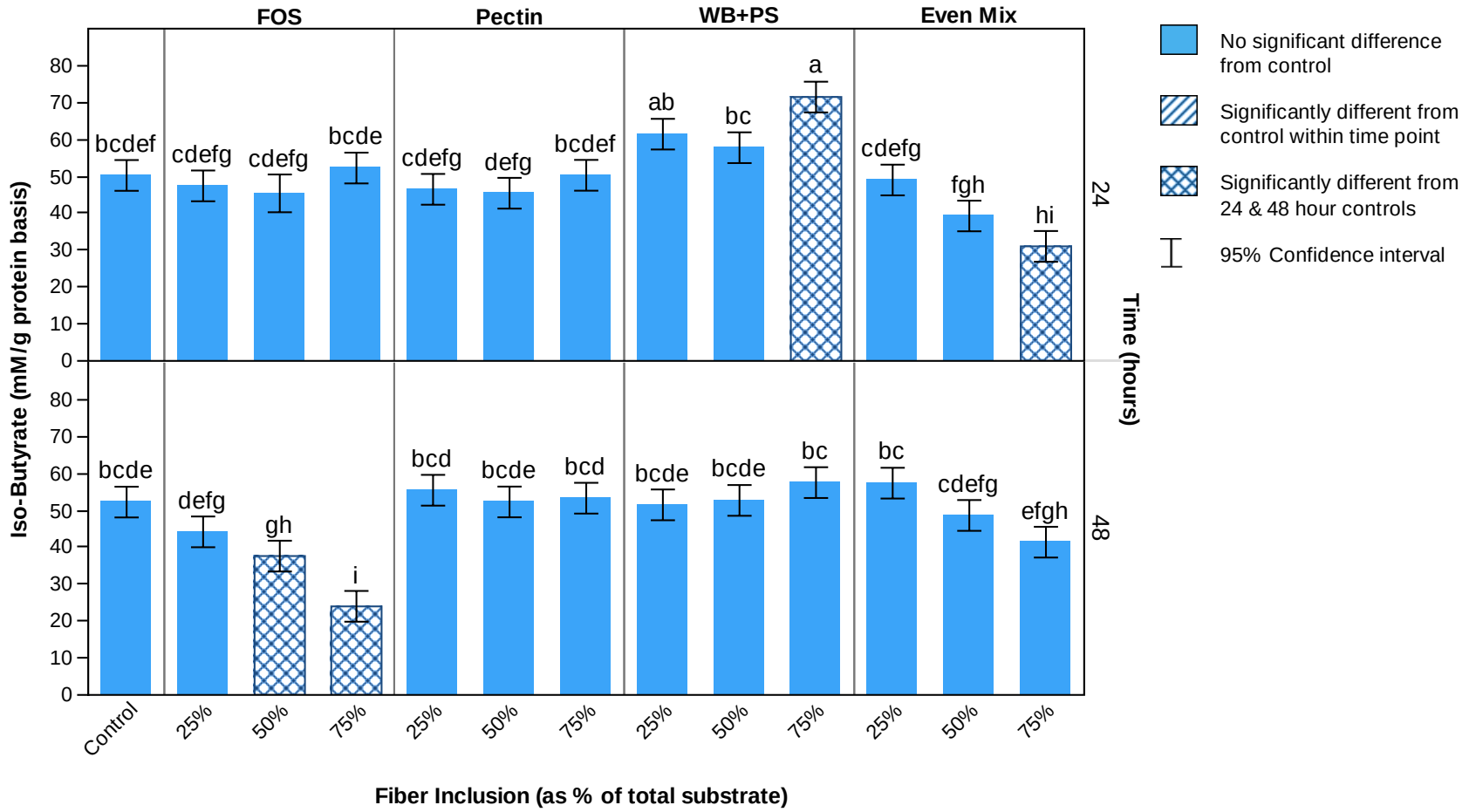

Figure 4.5. Least square mean iso-butyrate (mM/gram initial protein) after 24 and 48 hours of fermentation (secondary y-axis) of protein-fiber substrate mixtures (n=3), grouped by fiber substrate source (top). X-axis represents the initial proportion of fiber in the substrate mixture. 'Control' = 100% protein, 0% fiber substrate. Bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

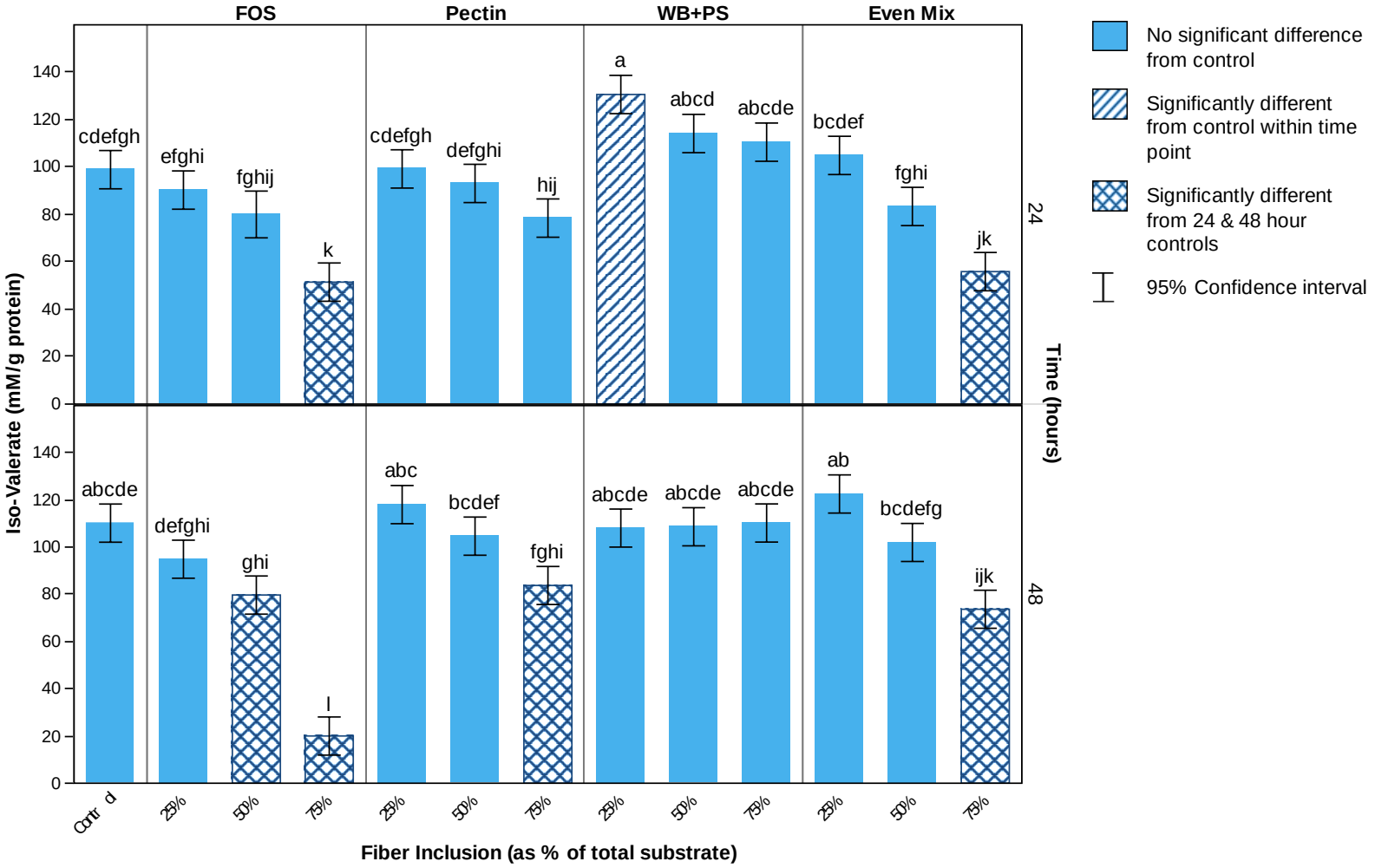

Figure 4.6. Least square mean iso-valerate (mM/gram initial protein) after 24 and 48 hours of fermentation (secondary y-axis) of protein-fiber substrate mixtures (n=3), grouped by fiber substrate source (top). X-axis represents the initial proportion of fiber in the substrate mixture. 'Control' = 100% protein, 0% fiber substrate. Bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

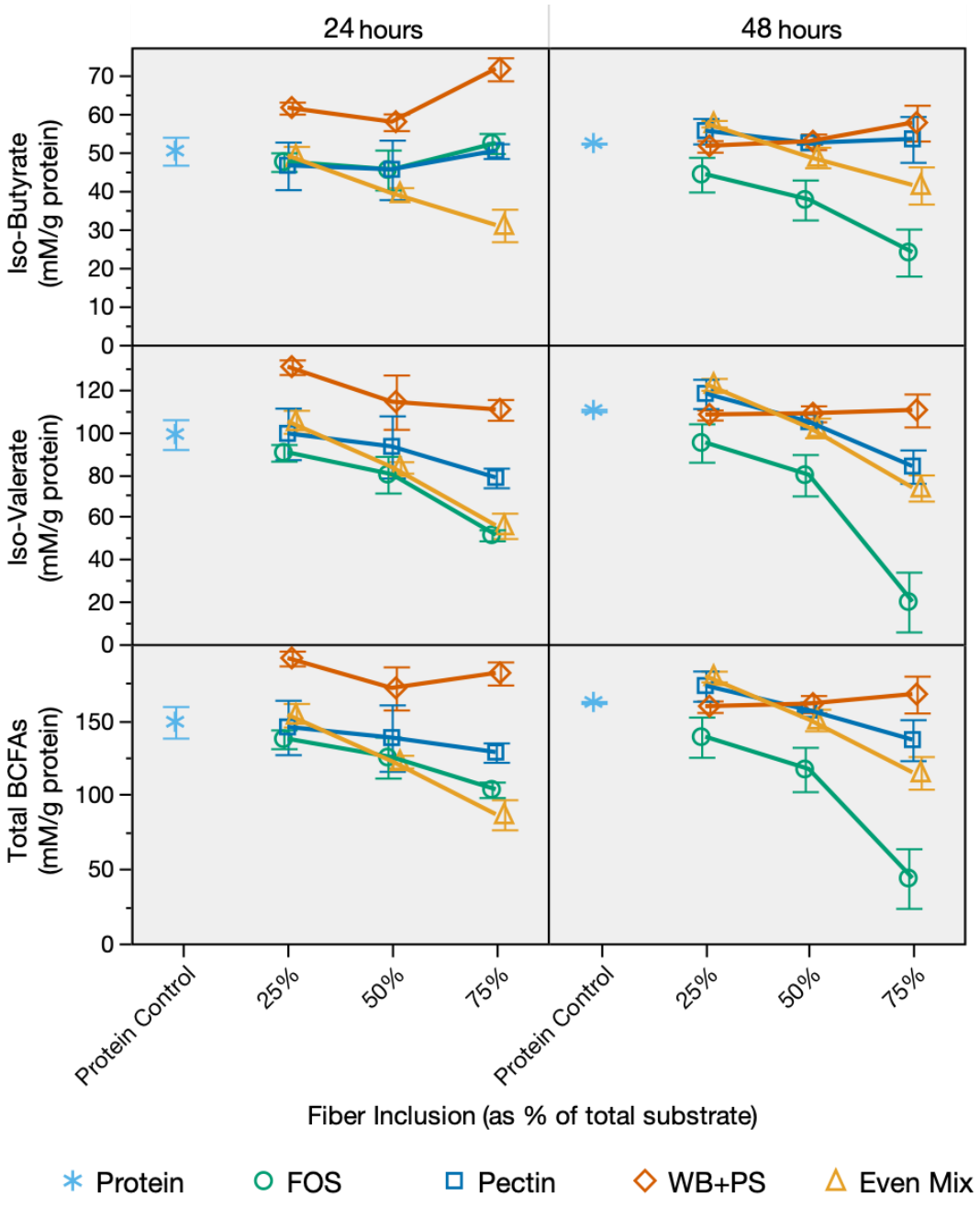

Figure 4.7. Mean iso-butyrate, iso-valerate, and total BCFA (mM/g protein) trends of protein-fiber substrate mixtures by fiber source and fiber inclusion level after 24 and 48 hours fermentation (n=3). Error bars = standard deviation. X-axis represents the percentage of fiber in the protein-fiber fermentation substrate mixture. 'Protein Control' = 100% protein, 0% fiber substrate. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

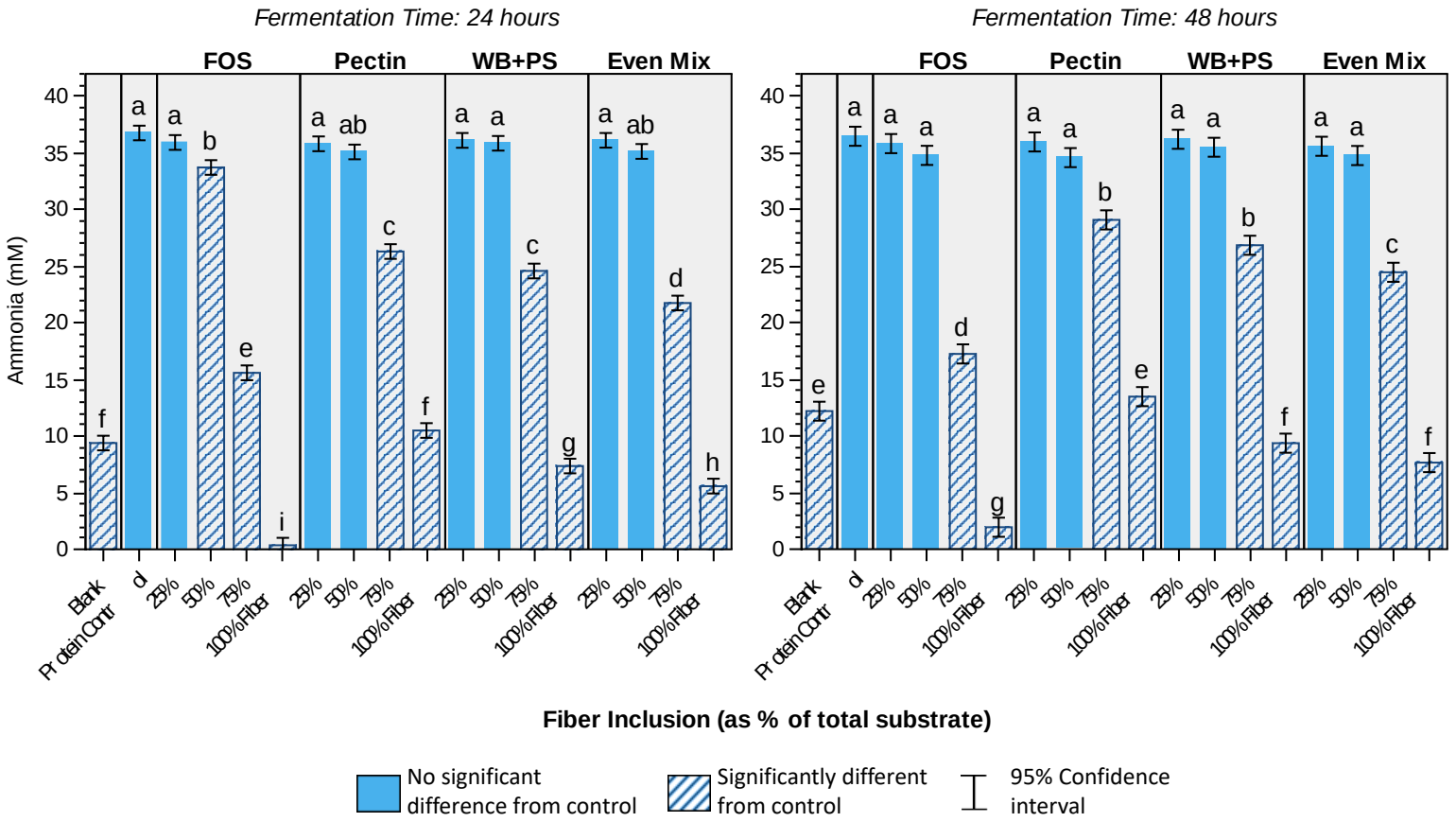

Figure 4.8. Least square mean ammonia concentration of protein-fiber substrate mixtures (n=3) after 24 and 48 hours fermentation, grouped by fermentation time and fiber substrate source (top). X-axis represents the percentage of fiber in the protein-fiber fermentation substrate mixture. 'Blank' = no substrate, inoculum only, 'Protein Control' = 100% protein, 0% fiber substrate. Within each time point, bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

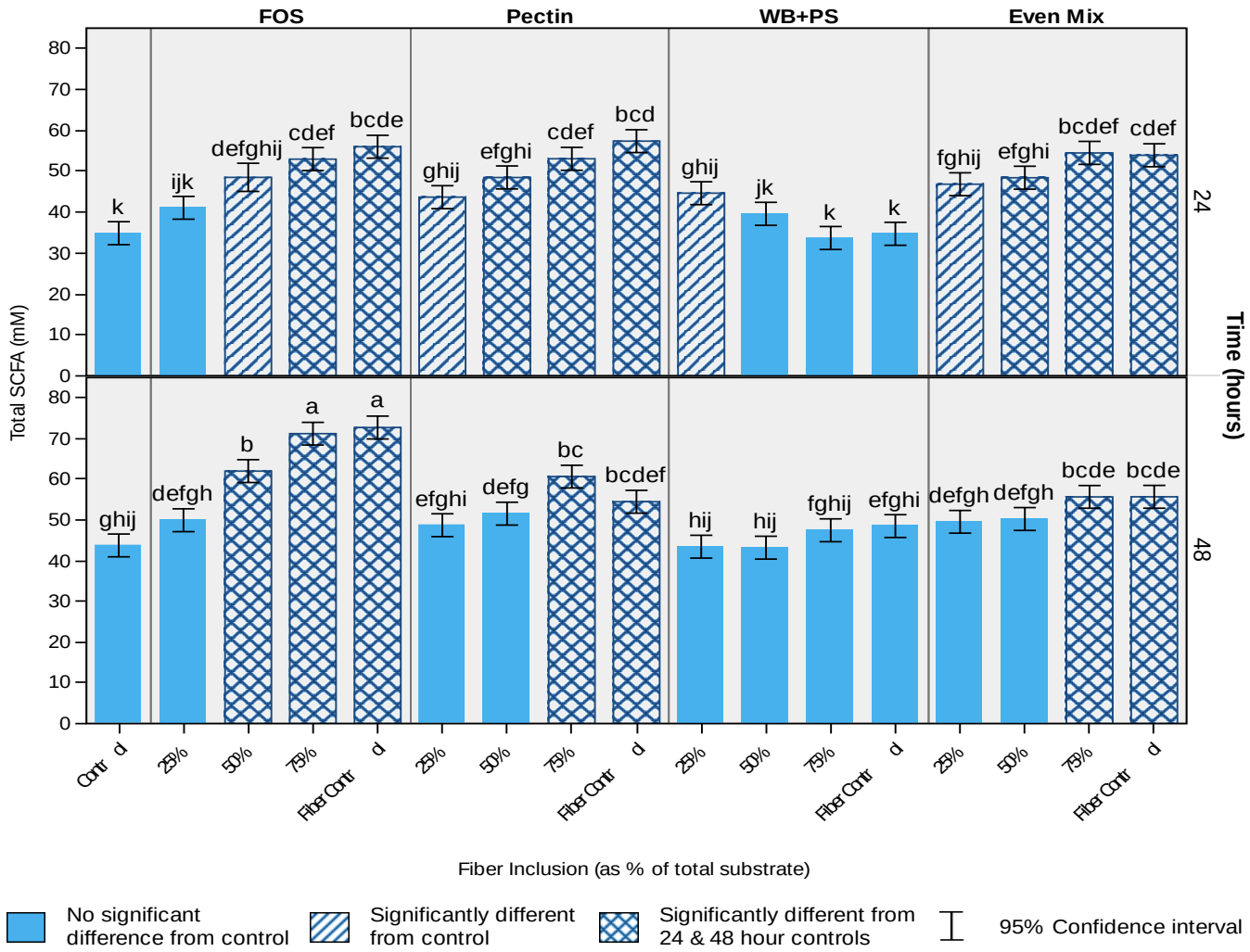

Figure 4.9. Least square mean total SCFAs produced after 24 and 48 hours of fermentation (secondary y-axis) of protein-fiber substrate mixtures (n=3), grouped by fiber substrate source (top). X-axis represents the initial proportion of fiber in the substrate mixture. 'Control' = 100% protein, 0% fiber substrate, 'Fiber Control' = 0% protein, 100% fiber substrate. Bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

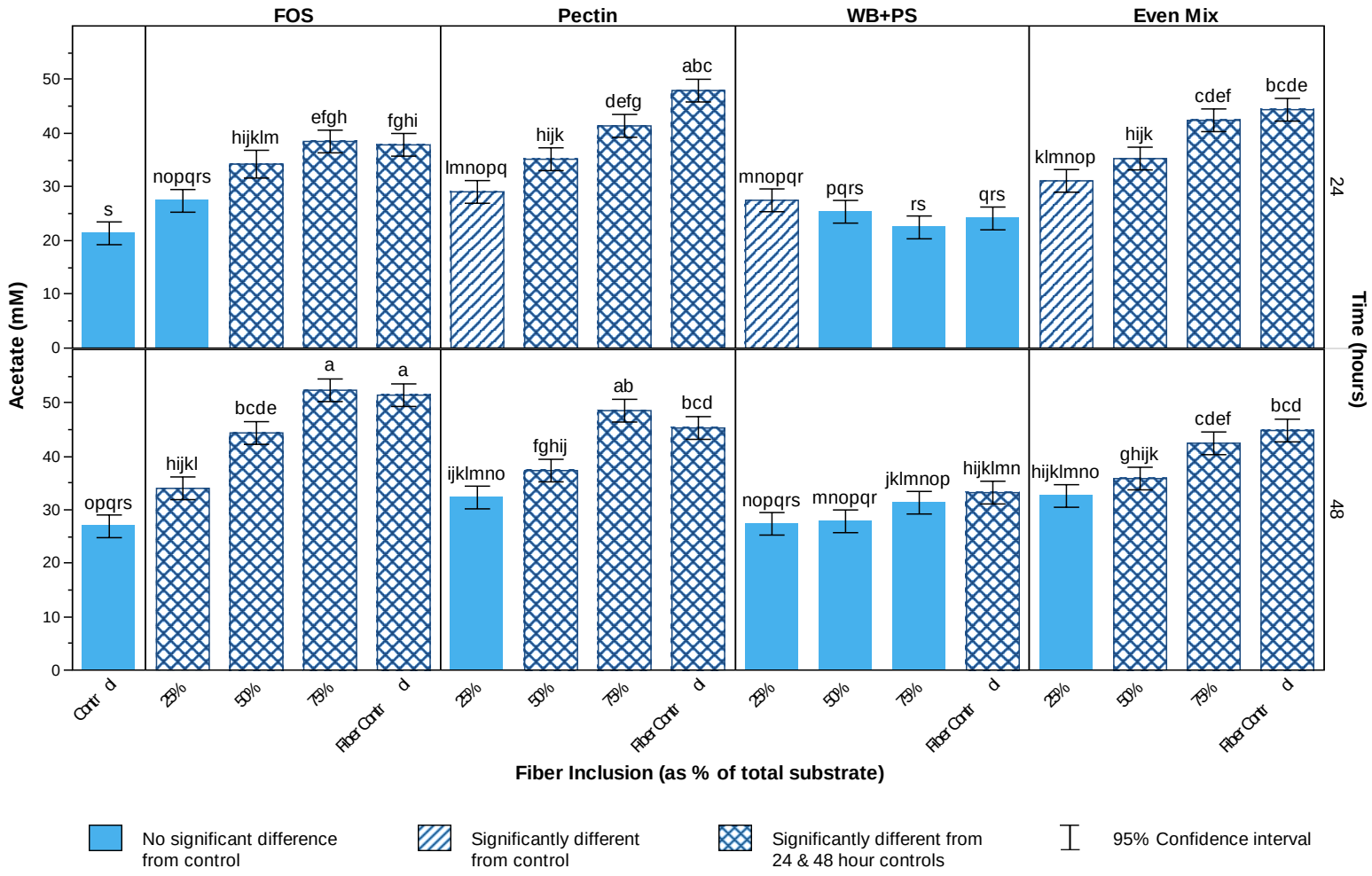

Figure 4.10. Least square mean acetate produced after 24 and 48 hours of fermentation (secondary y-axis) of protein-fiber substrate mixtures (n=3), grouped by fiber substrate source (top). X-axis represents the initial proportion of fiber in the substrate mixture. 'Control' = 100% protein, 0% fiber substrate, 'Fiber Control' = 0% protein, 100% fiber substrate. Bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

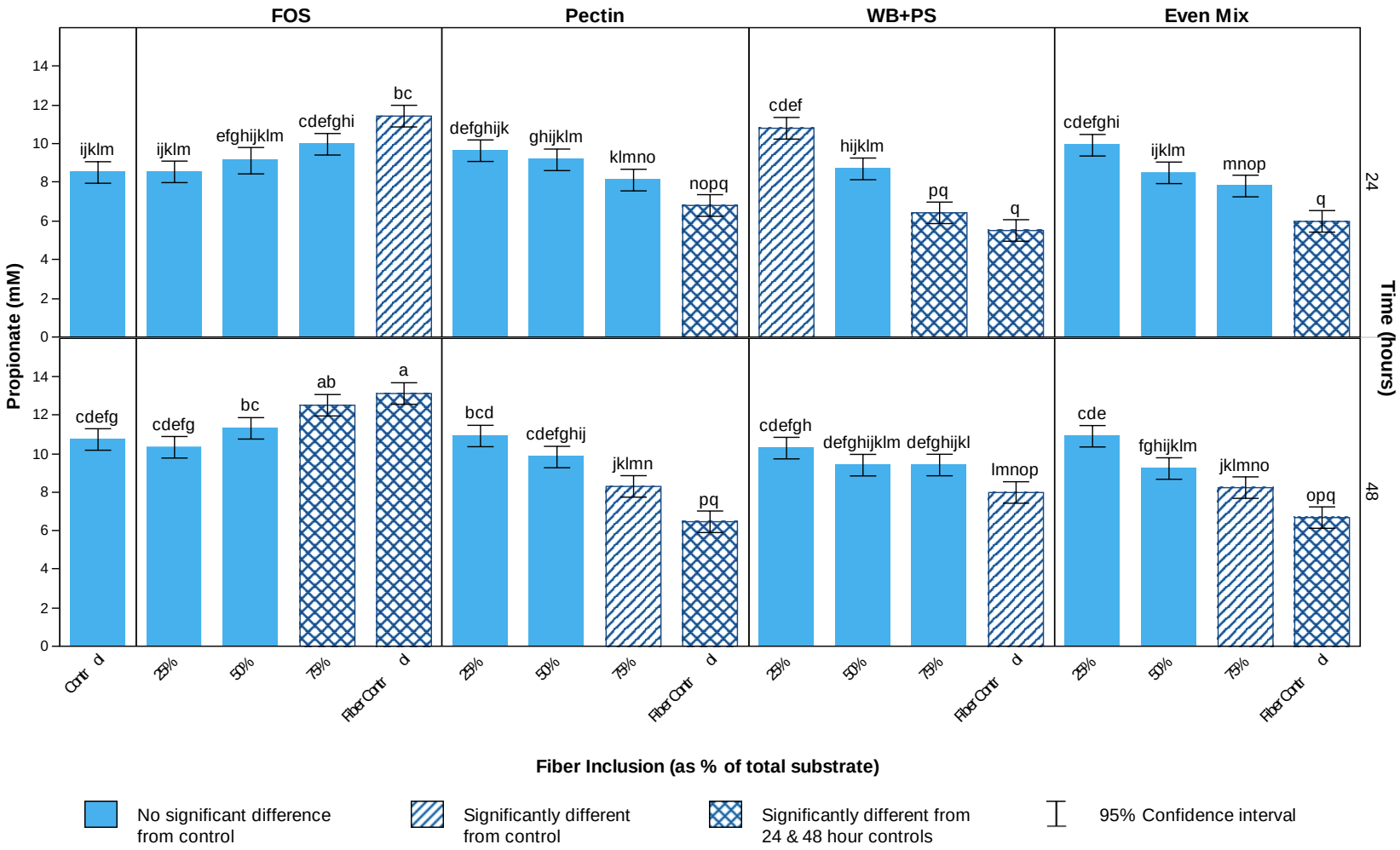

Figure 4.11. Least square mean propionate produced after 24 and 48 hours of fermentation (secondary y-axis) of protein-fiber substrate mixtures (n=3), grouped by fiber substrate source (top). X-axis represents the initial proportion of fiber in the substrate mixture. 'Control' = 100% protein, 0% fiber substrate, 'Fiber Control' = 0% protein, 100% fiber substrate. Bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

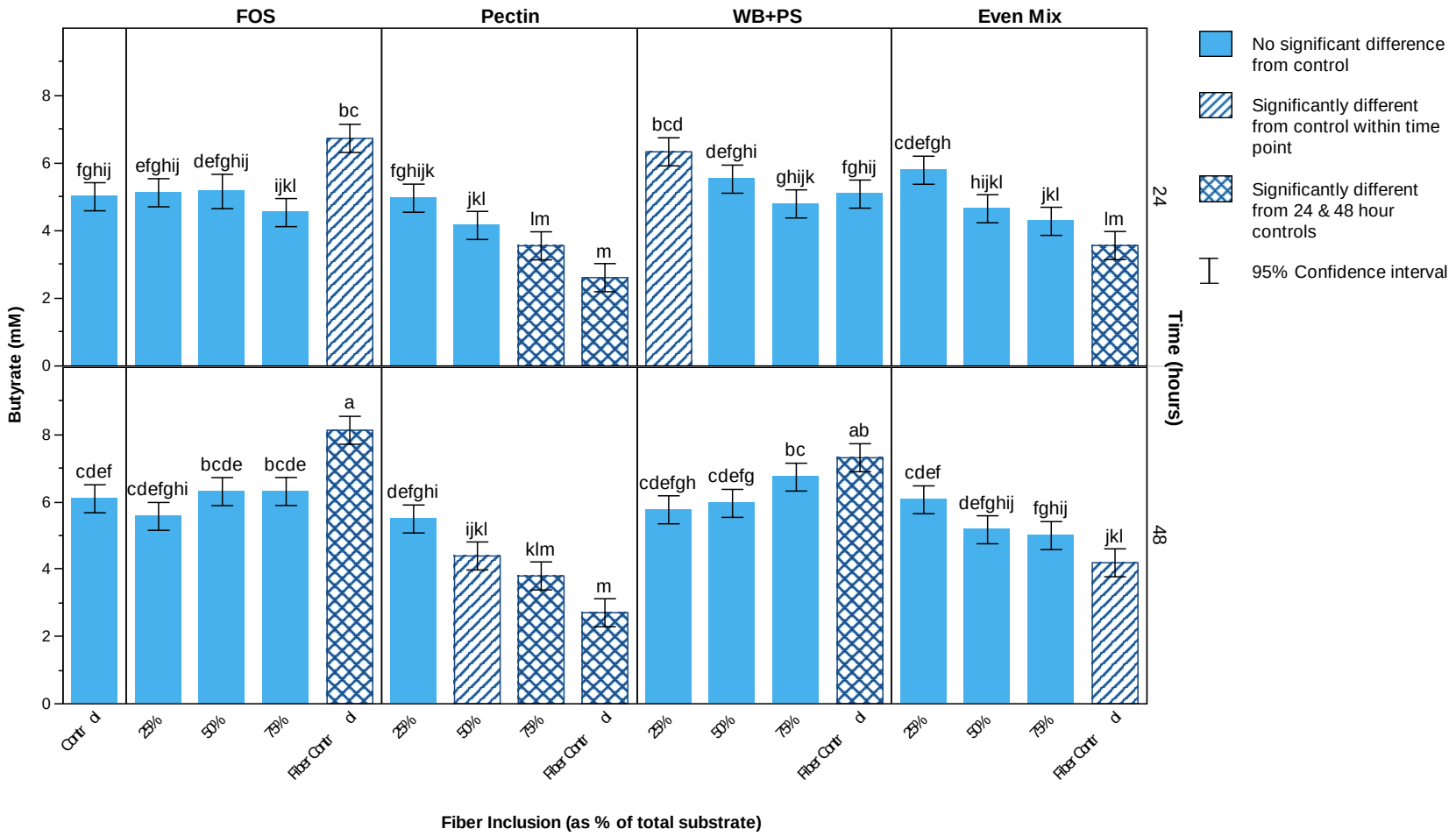

Figure 4.12. Least square mean butyrate produced after 24 and 48 hours of fermentation (secondary y-axis) of protein-fiber substrate mixtures (n=3), grouped by fiber substrate source (top). X-axis represents the initial proportion of fiber in the substrate mixture. 'Control' = 100% protein, 0% fiber substrate, 'Fiber Control' = 0% protein, 100% fiber substrate. Bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

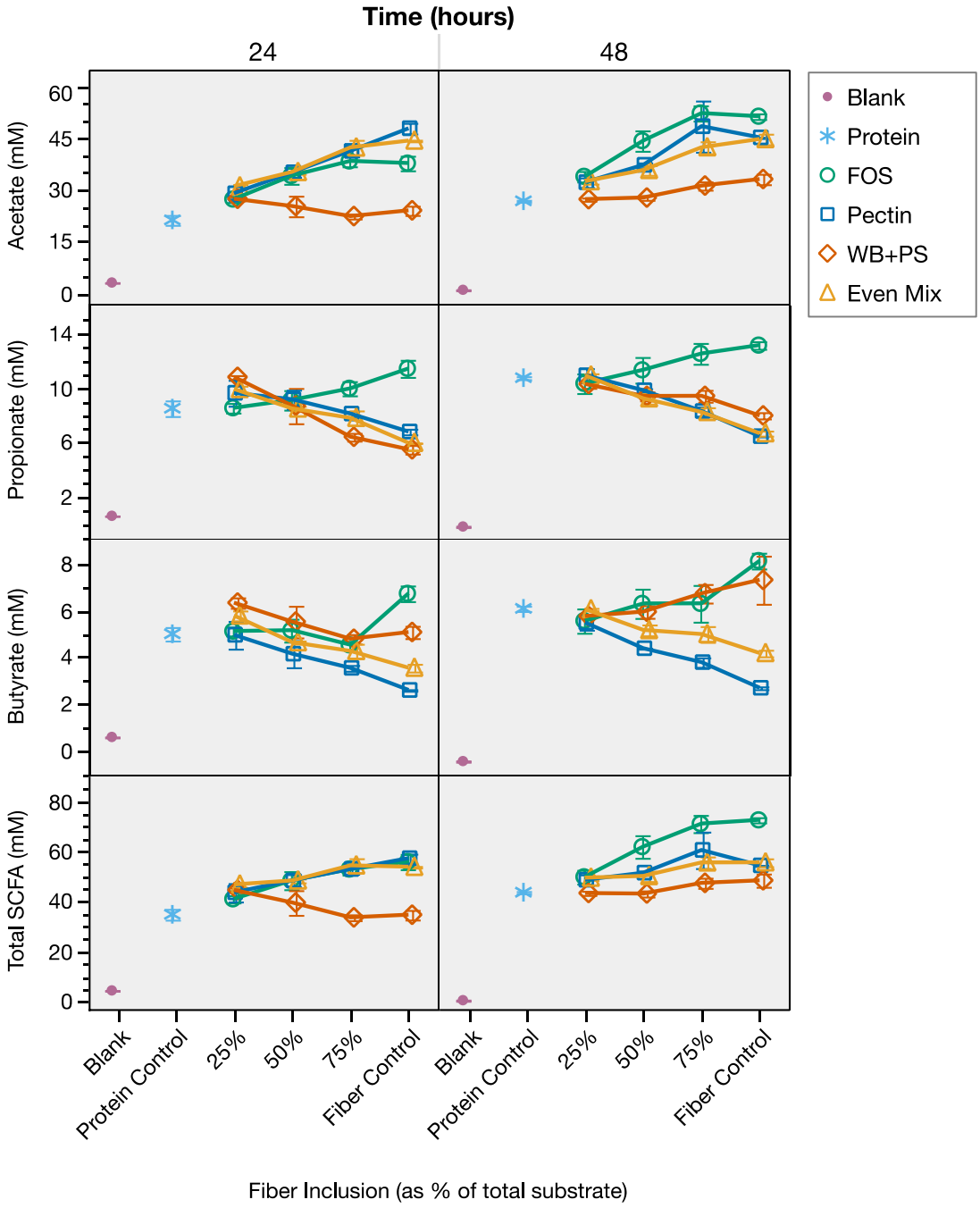

Figure 4.13. Mean acetate, propionate, butyrate, and total SCFA trends of protein-fiber substrate mixtures by fiber source and fiber inclusion level after 24 and 48 hours fermentation (n=3). Error bars = standard deviation. X-axis represents the percentage of fiber in the protein-fiber fermentation substrate mixture. 'Blank' = no substrate, inoculum only, 'Protein Control' = 100% protein, 0% fiber substrate, 'Fiber Control' = 0% protein, 100% fiber substrate, 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

CHAPTER 5. DISCUSSION

One of the primary purposes of this study was to examine the degree of effect that fiber source would have on metabolites produced via fermentation of protein-fiber substrates. Indeed, fermentation outcomes differed significantly among fiber sources. Therefore, the discussion first focuses on each individual fiber source and a comprehensive overview follows.

5.1 Discussion by Fiber Source

5.1.1 FOS Discussion

In a protein-FOS substrate mixture, it was hypothesized that the FOS portion would be fermented very quickly, producing voluminous SCFAs to significantly decrease pH, causing protein fermentation to be suppressed. It was also hypothesized that the suppression effect would be strengthened as the proportion of FOS increased. In support of our hypotheses, protein fermentation was suppressed the most in protein-FOS fiber substrates (Figure 4.4), and the suppression effect increased in magnitude as the proportion of FOS increased and pH decreased (Figure 5.2).

FOS had significantly lower pH and higher gas production values compared to the other fibers and the 100% protein control (Figure 4.3, Figure 4.2). This is in line with previous research finding FOS to have lower pH values and generate more gas than other common fast fermenting fibers, a mixture of fast fermenting fibers, or longer-chain fructans (Hernot et al., 2009; Probert & Gibson, 2002; Tuncil et al., 2017; Wiele, Boon, Possemiers, Jacobs, & Verstraete, 2007). At 24 hours, Figure 5.3 shows that protein-FOS mixtures had significantly lower pH values, indicative of high fermentative activity, yet total SCFAs were relatively similar to Pectin and Even Mix protein-fiber mixture samples at each inclusion level. The disparity between SCFAs and pH values is likely a consequence of the presence of an unmeasured intermediary metabolite such as lactate. Previous *in vitro* studies have shown FOS to produce lactate while pectin does not (Hernot et al., 2009; Wang & Gibson, 1993). This likely explains the similarity of total SCFA values. Additionally, lactate may have been formed via the fermentation of protein and/or an interaction effect of protein

and FOS. A recent *in vitro* study found casein, meat, and commercially available mycoprotein to generate measurable lactate at 6, 10, and 24 hours of fermentation (Wang et al., 2019). When these proteins were combined with a fiber mixture of short- and long-chain fructans, the lactate response increased by at least 1.5-fold. The difference between 24 and 48-hour total SCFAs of protein-FOS samples provides another piece of evidence pointing toward the presence of lactate. Within each fiber inclusion level, all FOS-containing samples had higher total SCFAs at 48 hours compared to 24-hour results, whereas this did not occur in any Pectin or Even Mix protein-fiber samples (and only occurred at the 75% inclusion level for WB+PS). Lactate is converted by microbiota to acetate, propionate, or butyrate, and as previously stated the fermentation of pectin has not been shown to produce lactate. The presence of lactate would therefore account for the incongruity of pH values compared to total SCFAs in the first 24 hours and the unexpected formation of more SCFAs in FOS samples during the second 24-hour period.

While it is unclear from this study precisely which metabolites of fermentation lead to the significant decrease in pH for FOS-containing samples, it is clear that low pH values likely had a strong influence in the suppression of protein fermentation when FOS was included. It is well-cited in the literature that protein/amino acid metabolism is hindered in relatively low pH environments (Cummings, 1981; Cummings et al., 1987; Gibson, McFarlan, Hay, & MacFarlane, 1989; Macfarlane, Gibson, Beatty, et al., 1992; Macfarlane, Gibson, & Cummings, 1992; Smith & Macfarlane, 1996, 1998). As shown in Figure 4.3, there was significant correlation between pH and fiber inclusion level for all tested fiber sources. However, the relationship between total BCFAs (mM/g protein basis) and fiber inclusion level was dependent on fiber source, with FOS having the highest positive correlation ($r = 0.8337$, $p < 0.0001$). pH and total BCFAs (mM/g protein basis) were most highly positively correlated for protein-FOS samples ($r = 0.7944$, $p < 0.0001$), whereas Even Mix and Pectin protein-fiber mixture samples were less so ($r = 0.6979$, $p = 0.0013$; $r = 0.5128$, $p = 0.0294$ respectively), and there was no significant correlation in WB+PS samples ($r = 0.2429$, $p = 0.3303$). To further support the likelihood that low pH likely caused the protein fermentation suppression in FOS samples, Figure 5.2 shows that even though FOS is well documented to be completely fermented within 6-12 hours (Hernot et al., 2009; Probert & Gibson, 2002; Tuncil et al., 2017; Wang & Gibson, 1993; Wiele et al., 2007) the suppression effect extended throughout the full 48 hour fermentation period. Research studies have shown that FOS

is capable of being fermented more quickly than longer chain fructans, more complex and less enzymatically accessible structures, and other fast-fermenting fibers such as galactooligosaccharides and polygalacturonans individually or in mixtures (Hernot et al., 2009; Tuncil et al., 2017; Wang & Gibson, 1993; Wiele et al., 2007). In this study, pH was measured at 0, 24, and 48 hours, but it is conjectured that the drop in FOS's pH was not only significant compared to that of the other fibers but also occurred at a much faster rate. Some evidence of this can be found by comparing total BCFAs (mM/ g protein) between the fibers when pH levels were statistically similar. For example, there was no significant difference between the pH of FOS (25) and the 50% inclusion levels of Pectin, WB+PS, or Even Mix samples. However, FOS (25) had lower total BCFAs (mM/g protein basis) than the 50% inclusion level of the other fiber sources [although only with significance for WB+PS (50) at 24 hours]. This suggests the pH dropped more quickly in the FOS-containing samples, and the suppression of protein fermentation occurred more quickly and in greater force than in the other tested fibers. Once the pH made protein fermentation less favorable, microbiota may have shifted to utilizing the protein substrate as a nitrogen source for bacterial cell growth (Cummings, 1981; Davila et al., 2013).

In vivo, FOS-inclusion would likely have a more limited impact of lesser duration on suppressing protein fermentation. Movement through the colon cannot be accounted for in a static *in vitro* batch method such that was used in this study. *In vivo,* the fermentation of FOS would most likely suppress protein fermentation in the immediate colonic environment. Since FOS ferments quickly, only the ascending colon and possibly a portion of the transverse colon would get the benefit of lower pH and subsequent suppression of protein fermentation. However, once FOS was completely fermented, the remaining proteinaceous substrate would continue to move along the colonic tract, toward the distal colon where pH levels are higher.

Compared to the 100% protein control, FOS as the sole substrate produced significantly more butyrate, although even a small addition of protein greatly reduced butyrate concentrations to levels lesser than or equal to that of the 100% protein control (Figure 4.12). This is important because, *in vivo,* some protein is always available for fermentation in the colon from endogenous sources even when on an extremely low protein diet (Chacko & Cummings, 1988). *In vitro* microbiome-related studies that do not include proteinaceous substrate with FOS may over-

estimate projected *in vivo* butyrate production. It may be wise to include protein *in vitro* to better mimic the *in vivo* environment.

5.1.2 Pectin Discussion

High-protein low-carbohydrate diets have been shown to decrease total SCFAs, fecal butyrate, butyrate proportion, and butyrate-producing bacteria (Duncan et al., 2007; Russell et al., 2011). We hypothesized inclusion of pectin fiber would counterbalance outcomes of protein fermentation by increasing both total SCFAs and butyrate, promoting butyrate-producers, and suppressing generation of ammonia.

As predicted, and shown in Figure 4.9, inclusion of Pectin increased total SCFAs compared to the 100% protein control, particularly within the first 24 hours of fermentation. This was almost entirely from an increase in acetate, which was predicted in our hypothesis, and is in agreement with multiple previous studies in which pectin was found to preferentially increase acetate (Bang et al., 2018; Chen, 2016; Ferreira-Lazarte et al., 2019; Pirman et al., 2007; Reichardt et al., 2018).

We had hypothesized Pectin inclusion would increase butyrate concentration as well as butyrate proportion, which would be indicative of promotion of microbial butyrate-producers. Instead, quite surprisingly, pectin was not very butyrogenic in this study. The 100% Pectin fiber control generated the least butyrate of all samples in this study (except the blank); in fact, Pectin had significantly less butyrate than the 100% protein control and inclusion of Pectin suppressed butyrate (Figure 4.12). As shown in Figure 5.1, the molar proportion of butyrate also fell, giving the appearance that Pectin inclusion actually had an inhibitory effect of butyrogenic bacteria instead of the anticipated promotion effect.

The lack of butyrogenic effect may be partially explained by the pH used in our study. The effect of pH on SCFA outcomes has been tested in several studies, but our pH values [7.48 ± 0.3 for Pectin samples (Figure 4.3)] were higher than those found in the literature. In an *in vitro* study, fermentation of a mixture of pectin and other dietary fibers at pH 6.5 suppressed butyrate production when compared to fermentation at pH 5.5 (Walker et al., 2005). In contrast, a later study using the same mixture of dietary fibers found that butyrate proportions were unchanged at

either pH 6.4 or 5.9 (Belenguer et al., 2007). Recently in a large, robust *in vitro* study, fermentation of apple pectin at pH 6.5 suppressed butyrate concentrations and proportions compared to fermentation at pH 5.5 (Reichardt et al., 2018). Using apple pectin as the sole carbon source, the abundance of *Faecalibacterium prausnitzii*, a major butyrate-producer in the large intestine, increased sharply when the pH was lowered from 6.9 to 5.5, possibly because *F. prausnitzii* spp. were more acid-tolerant and able to out-compete the *Bacteroides* spp. that had dominated at a higher pH (Chung et al., 2016). Some major butyrate-producing bacteria such as *F. prausnitzii* and *Roseburia* spp. require acetate to synthesize butyrate (Duncan et al., 2004; Louis & Flint, 2017). Therefore, the high pH in our study likely favored bacteria that are not large butyrate producers. Had the pH decreased more during fermentation, perhaps butyrate producers that utilize acetate would have been favored, giving rise to the expected butyrate production from pectin.

The use of Pectin in a protein-fiber mixture did not inhibit protein fermentation. As shown in Figure 4.4, when compared to the 100% protein control, Pectin inclusion did not have a significant effect on suppression of BCFA production (mM/g protein basis) at any inclusion level or time point. Furthermore, Pectin inclusion suppressed the concentration of ammonia only when Pectin made up the majority of the substrate (Figure 4.8). Studies have shown that when proteolytic fermentation is reduced, ammonia concentration is also reduced. An *in vitro* study using a dynamic GI simulator found citrus pectin to slightly reduce ammonium concentration during a 14-day chronic feeding period (Ferreira-Lazarte et al., 2019), though a proteinaceous substrate was not part of the study. Our ammonia concentration results for the 100% Pectin fiber control contradict those of the aforementioned study, in which pectin slightly but significantly reduced ammonium concentrations compared to the blank. In our study, the mean concentration of ammonia for the 100% Pectin fiber control was not significantly different from the blank (blank = 10.8 ± 1.6, 100% Pectin fiber control = 12.0 ± 1.7), whereas the other 100% fiber controls had lower concentrations compared to the blank, ranging from 1.2 – 8.4 mM. Furthermore, when comparing 100% fiber controls, Figure 4.8 shows Pectin had the highest ammonia concentration of the fibers with significance. This agrees with several *in vivo* studies that did not find pectin supplementation beneficial for ammonia inhibition. When pectin was supplemented daily for 3 weeks in humans (n=6), there was a significant increase in stool ammonia concentrations, and during *in vitro* fermentation the rate of ammonia formation increased (Mallett et al., 1988). This could indicate

pectin supplementation increased protein fermentation in the large intestine, however the supplementation of pectin may have also lowered the digestibility of protein in the small intestine, resulting in a greater amount of fermentable protein delivered to the colon. A diet study using pigs found ammonia concentration was not affected by supplementation with pectin, sugar beet pulp, or soya bean hulls (Hansen, Chwalibog, & Tauson, 2007). In another diet study, when diet interaction effects of dietary protein (casein or potato) and dietary fibers (cellulose, potato starch, or pectin) in pigs were examined, protein source, but not dietary fiber supplementation, affected colonic ammonia concentrations (Taciak, Barszcz, Święch, Tuśnio, & Bachanek, 2017).

In conclusion, inclusion of Pectin in a protein-fiber substrate led to higher total SCFA production but did not significantly alter protein fermentation. While it did suppress ammonia at 75% inclusion, this was likely an effect of low protein availability rather than the specific inclusion of Pectin fiber. When it comes to increasing butyrate and/or decreasing protein fermentation, our study did not find inclusion of Pectin to be effective.

5.1.3 WB+PS Discussion

The study results partially support our hypothesis that inclusion of WB+PS in a protein-fiber substrate mixture would have a sustained butyrogenic effect, as well as inhibit protein fermentation and ammonia concentration levels. Inclusion of WB+PS in a protein-fiber substrate mixture did not increase cumulative total SCFAs over 48 hours, inhibit BCFA production (mM/g protein basis), nor out-perform the other tested fibers in suppressing ammonia production, however it did have a positive effect on butyrate production.

WB+PS (25) was the only tested fiber and inclusion level to increase butyrate concentration compared to the 100% protein control (Figure 4.12), and interestingly there was a concurrent increase in protein fermentation (Figure 4.4). When in a mixture with protein, WB+PS was also the only tested fiber in which increasing fiber proportions led to increasing butyrate on a molar proportional basis (Figure 5.1).

Compared to the 100% protein control, as shown in Figure 4.4, WB+PS (25) produced more BCFAs (mM/g protein basis). However, this only occurred in the first 24 hours. After the second

24-hour period it was similar to the 100% protein control. In agreement with our results, it was previously demonstrated BCFAs were more rapidly formed in the presence of carbohydrate (as gelatinized soluble starch) during *in vitro* fermentation of peptone water, yet cumulative BCFAs over time remained constant (Macfarlane, Gibson, Beatty, et al., 1992). Although in contrast, a later study found starch slowed the rate of BCFA production (Smith & Macfarlane, 1998). Perhaps more importantly though, the Smith and Macfarlane (1998) study found the most influential factor for rate and total accumulation of BCFAs was pH, with a lower pH (5.5) inhibiting BCFAs quite considerably. As discussed previously in 5.1 above, pH was likely the determining factor for inhibition of protein fermentation due to a quick and substantial drop in pH. However, in the case of WB+PS (25), pH was not a significant factor since it was the only sample compared to the 100% protein control with a similar and relatively high pH. The most likely conclusion is that when pH level remains relatively constant, low level WB+PS inclusion in a protein-fiber substrate increases the fermentative rate of protein but does not alter the cumulative quantity of protein fermented.

There was a concurrent increase in butyrate production in conjunction with faster protein fermentation for 24-hour WB+PS (25) (Figure 4.12). BCFA increases indicate more protein was utilized for energy. Furthermore, WB+PS (25) had significantly more butyrate than its 100% fiber control. This result, combined with WB+PS (25)'s increased protein fermentation, indicates that enhanced butyrate production is likely a result of protein fermentation. However, some studies have reported an interaction effect of protein and type II resistant starch (RS2) wherein protein encourages greater microbial utilization of RS2 to increase butyrate concentrations (Le Leu et al., 2006; Morita et al., 1998). In the first of these studies, protein was pre-treated to create a digestion-resistant protein, termed resistant protein, ensuring greater amounts of protein would enter the cecum of rats (Morita et al., 1998). There was a tremendous increase in cecal butyrate concentration leaping from 25.5 ± 3.9 µmol on a standard casein diet supplemented with RS2 to 111.1 ± 16.3 µmol when 6% resistant protein was used. Unfortunately, the study did not include direct markers of protein fermentation such as BCFAs. However, it was concluded resistant protein led to more microbial utilization of RS2 because fecal nitrogen increased at the same time that fecal starch excretion decreased. It is interesting that in our study Pectin and 100% Even Mix fiber controls resulted in less butyrate than the 100% protein control. Clearly, while the presence of protein may promote carbohydrate utilization, protein itself also had butyrogenic potential. A later

study using RS2 and resistant potato protein in rats induced with colon carcinoma (Le Leu et al., 2006) showed similar results to the Morita et al. study. However, the Le Leu et al. (2006) study showed that the interaction effect of RS2 and resistant protein on butyrate concentration in the cecum did not carry through to the proximal or distal regions of the colon. In contrast to our study, RS2 was found to lower total BCFAs throughout the colon regardless of dietary protein used. Our tested fibers *in vitro* did not largely attenuate formation of BCFAs, and WB+PS actually increased BCFAs (mM/g protein basis). Furthermore, although the Le Leu et al. (2006) study found higher butyrate concentrations with the resistant protein diet, alarmingly it also promoted tumorigenesis in the small intestine which was not prevented by RS2 supplementation. The reason this occurred in the small, but not the large intestine, requires further research, but for now it serves as a reminder that caution is warranted regarding any potential benefits of using protein to increase butyrate in the gut.

Similar to results of some *in vivo* animal studies, the 100% protein control in our study produced more butyrate than some fibers (Even Mix and Pectin), suggesting that protein can be a butyrogenic agent. For rats on a high or low protein diet, a comparison of dietary protein sources found significantly higher cecal butyrate concentrations when on soy protein compared to casein or whey regardless of the proportion of protein in the diet (Toden et al., 2007). Soy protein is less digestible than casein or whey (Almeida, Monteiro, Costa-Lima, Alvares, & Conte-Junior, 2015; Windey et al., 2012), so it is reasonable to assume more soy protein reaching the colon daily was implicit with butyrate concentration increases, however the significance of protein source specificity remains unclear. Early work involving *in vitro* batch protein fermentations of casein and bovine serum albumin found the proteins produced similar molar ratios of acetate but differed in propionate, butyrate, and BCFA ratios (Macfarlane, Gibson, Beatty, et al., 1992). In the above-mentioned Toden et al. study, adding RS increased butyrate, although rats on the soy protein diet still had the highest butyrate levels overall, and no interaction effect was found between RS and protein. This agrees with our study and may explain why WB+PS, which contained 50% raw starch, was the only tested fiber to increase butyrate concentration when combined with protein (of which 15% was soy protein). Finally, another study found that when compared to a casein protein diet, pigs on a potato protein (i.e., resistant protein) diet had increased butyrate and protein fermentation products (p-cresol, indole, iso-valerate and amines) (Taciak et al., 2017), which again connects

increased colonic dietary protein with butyrate. As with the previous study, when RS was added to the diet there was no evidence of an interaction effect of protein and RS on butyrate response.

Of course, the WB+PS fiber contained 50% wheat bran in addition to the resistant starch. Only the 25% inclusion level of WB+PS, corresponding to a high-protein low-fiber fermentation ratio, had higher total SCFAs and BCFAs (mM/g protein basis) than the 100% protein control (Figure 4.9, Figure 4.4). Similarly, pigs on a high-protein low-wheat bran diet were found to have higher SCFAs and BCFAs than those on a high-protein high-wheat bran diet (Pieper et al., 2012). Although, in contrast with our results, the study also found ammonia concentration was significantly decreased on the high-protein low-wheat bran diet. While, in our study, there was not a significant difference in ammonia concentration until the 75% inclusion level of WB+PS (Figure 4.8), it is difficult to compare *in vitro* ammonia results with that of an *in vivo* study since the natural absorption of ammonia through the epithelium cannot be accounted for in an *in vitro* study.

There is, of course, difficulty in comparing WB+PS outcomes to studies using only wheat bran or only resistant starch since mixed dietary fibers may have different fermentation profiles than their individual components (Tuncil et al., 2017). Combined wheat bran and resistant starch supplementation effect on fermentation profiles has been examined in several *in vivo* studies. A pig study found that when RS2 was paired with wheat bran it inhibited cecal fermentation, thereby shifting fermentation toward distal regions and increasing distal butyrate concentrations (Govers, Gannon, Dunshea, Gibson, & Muir, 1999). Additionally, the interaction of RS2 and wheat bran significantly decreased ammonia concentrations along the entire length of the colon. This is in contrast to our ammonia concentration results wherein WB+PS, comprised of ½ wheat bran and ½ resistant starch, did not perform as well as FOS or the Even Mix (composed of 16.7% wheat bran and 16.7% potato starch). A human diet study found supplementation of wheat bran and resistant starch (primarily as type II RS) to significantly decrease markers of colonic protein fermentation activity (Muir et al., 2004). The diet increased fecal butyrate concentrations and lowered fecal pH, ammonia, and iso-valerate (the BCFA typically found in highest proportion) compared to both the control diet and a diet supplemented only with wheat bran. This is in contrast with our results, which found an increase or no significant effect on BCFAs, though as mentioned previously, when the proportion of WB+PS to protein was high (i.e. the 75% inclusion level),

ammonia concentrations fell significantly compared to the 100% protein control. Finally, in a recent study, the effect of heat processing on colonic fermentation of either wheat bran and potato starch or peas in the diet of pigs was examined (Nielsen, Jørgensen, Knudsen, & Lærke, 2017). Of particular interest was that for pigs on the raw wheat bran and potato starch diet, casein protein was used which is highly digestible (only 5 grams per day were found to reach the colon). However, since peas contain a high level of protein, casein was removed entirely from the study diet in order to maintain similar dietary protein composition levels. Furthermore, uncooked protein is less digestible, so 27 grams per day of protein was delivered to the colon in pigs on this diet. Therefore, the wheat bran and potato starch diet used in the study was very similar to our study's 100% WB+PS fiber control, and pigs on the pea protein diet are comparable to that of our 100% protein control. Under these assumptions, our results mostly concur with findings from the *in vivo* pig study which showed no significant differences in total SCFAs, acetate (at 24 hours), or butyrate.

5.1.4 Even Mix Discussion

Based on evidence that fast-fermenting fibers are slower fermenting in a mixture, we combined our FOS, Pectin, and WB+PS fibers to test the hypothesis that the mixture would have a sustained suppression effect on protein fermentation due to a longer fermentation time and microbial preference for carbohydrates over protein for energy.

Similar to FOS, but unlike Pectin or WB+PS, 75% inclusion of Even Mix had a significant inhibitory effect on protein fermentation compared to the 100% protein control that continued throughout the 48-hour period (Figure 4.4). As shown in Figure 5.2, the protein fermentation suppression effect was unlikely to be related to pH, suggesting, as hypothesized, that carbohydrate preference may have played a role, especially since the higher pH levels are not considered inhibitory for protein fermentation. Additionally, Even Mix more strongly inhibited ammonia formation at the 75% level compared to Pectin and WB+PS.

A 1999 *in vitro* study[7], which could not be procured directly but was detailed within literature by the author's same lab group (Muir & Yeow, 2000), closely resembled our Even Mix protein-fiber

[7] Lim, J. (1999). *Diet and in vitro fermentation-dependent parameters relevant to colon cancer risk: Effects of protein, resistant starch and dietary fibre.* (Honours Thesis). Monash University.

substrate mixture. The Lim (1999) study used cooked red meat that was fermented for 24 hours with or without a carbohydrate mixture of resistant starch, wheat bran, and fruit and vegetable fiber isolates. In agreement with our study results, inclusion of the carbohydrate mixture decreased pH and increased acetate. However, the similarity between the studies diverge from there. The Lim (1999) study found that red meat combined with the carbohydrate mixture increased propionate and butyrate and decreased ammonia compared to red meat alone. We found no significant difference at any fiber inclusion level for butyrate concentration and a slight decrease for propionate when compared to the 100% protein control. As mentioned previously in the Pectin discussion (5.1.2), differences in fermentation pH may explain differences in our results. The meat protein study reported pH values of 6.2 for the blank and 100% protein control and pH 5.5 for the meat plus carbohydrate samples after 24 hours fermentation. In contrast, our pH values were 8.4, 7.9, and 7.2-7.7 for the blank, 100% protein control, and Even Mix-containing samples, respectively. The protein source may also be a factor in result differences as discussed previously (5.1.2). However, the literature (Muir & Yeow, 2000) states similar results were obtained when the Lim (1999) study was repeated in a separate experiment with different sources of protein (fish, chicken, and tofu).

Conceivably, human diet studies in which subjects consume their normal diets loosely relate to the Even Mix fibers used in our study due to the common (albeit typically low) inclusion of sources of FOS, pectin, wheat bran, and resistant starch in everyday foods of the Western diet. For example, FOS is found in onions and artichokes, pectin is naturally occurring in all fruits and vegetables, wheat bran is common in breakfast cereals, and raw potato starch is often used as an anti-caking agent in items such as shredded cheese. As stated previously, based on estimates and a Western diet, similar amounts of protein and dietary fiber reach the colon, which corresponds to the 50% inclusion level in our study. A 1997 study tested the effect of a protein supplement on human colonic bacterial metabolite formation (Geypens et al., 1997). Volunteers ate their normal diet for 1 week then for the following week they took a daily protein supplement at breakfast, lunch, and dinner, totaling an extra 58 grams of daily protein (primarily as whey, casein, and lactalbumin). The supplemental diet in week 2 relates most closely with the 25% Even Mix-fiber inclusion level of our study. Similar to our study, propionate, BCFAs, and ammonia increased with protein supplementation. However, in contrast, total SCFAs increased on the protein supplement diet

whereas there were no differences in our study between the 25 and 50% inclusion level of Even Mix.

In summary, Even Mix supplementation with protein produced more total SCFAs, primarily as acetate, but decreased beneficial butyrate. It was hypothesized inclusion of Even Mix fiber would have a lasting inhibitory effect on protein fermentation. At most levels it was not effective at suppressing formation of protein fermentation products at either time points. Yet, our results do suggest that when the proportion of Even Mix fibers to protein was high it was one of the most effective and sustaining inhibitors of protein fermentation (Figure 4.4). In translation, this essentially reiterates what has long been espoused: eat more vegetables and grains.

5.2 The Bigger Picture: Some General Trends and Key Takeaways

The purpose of this thesis study was elucidation of how substrate ratios in protein-fiber mixtures would affect protein fermentation and metabolites (specifically as BCFAs and ammonia), as well as examining the degree to which fiber source may influence these outcomes. The fundamental idea was aimed at incorporating dietary fibers to inhibit and/or counterbalance potentially negative outcomes of protein fermentation. It was reasoned that protein fermentation would be reduced when more fermentable dietary fiber was available due to gut bacterial preference for carbohydrates as an energy source. It was further speculated that specific fibers could be used to hinder protein fermentation and/or its products, which would depend on promoting the bacterial groups that generally respond to different fibers. For instance, FOS is used by many different types of bacteria, though it does not support well the mucosal Clostridia that contain many of the major butyrate producers. Pectin is perhaps utilized by a less diverse number of gut bacteria with promotion of the gram negative [-] Bacteroidetes, though also other Firmicutes including *F. prausnitzii*. WB-PS is an insoluble fermentable fiber mixture that has been shown to preferentially support the mucosal Clostridia groups, and the mixture of the fibers was hypothesized to slow fermentation of the fast-fermenting FOS and Pectin to possibly be effective to reduce protein fermentation at later incubation time. Finally, since proteinaceous substrate is ubiquitous in the gut, we were curious how its presence may change fiber fermentation profiles.

Firstly, data for our tested fibers affirmed several well established and accepted findings regarding gut microbial fermentation of dietary fibers. For most tested fibers in the protein-fiber mixture, rising proportions of dietary fiber related to increased accumulation of total SCFAs (Figure 4.13), increased gas production, and decreased pH (Figure 4.1). When fiber was the sole substrate, BCFA concentrations were virtually zero (i.e., under detection limits) (Table A.1), SCFA molar ratios (Figure 5.4, Table 5.1) were consistent with established values (Macfarlane, Gibson, & Cummings, 1992), and ammonia concentrations (Figure 4.8) were extremely low. Furthermore, most of the 100% fiber controls significantly reduced ammonia compared to the blank. With the exception of Pectin, the 100% fiber controls had significantly less ammonia than the blank, making it clear that on its own, when not in a protein-fiber mixture, some fibers can decrease ammonia concentrations likely due to lower pH or utilization of ammonia as a nitrogen source for microbial growth.

The disruption of protein fermentation by dietary fibers was more complex than the idea that simple availability of fermentable carbohydrates would suppress protein fermentation due to microbial preference for carbohydrates over proteins for energy. In protein-fiber substrate mixtures, total BCFAs (mM/g protein basis), which were used as a measure of protein fermentation, were relatively stable and not easily influenced by increasing the proportion of dietary fiber (Figure 4.4, Figure 4.7). Indeed, only half of the tested dietary fibers (FOS and Even Mix) inhibited protein fermentation, and the magnitude of the suppression effect was relatively small, requiring dietary fiber to make up at least half of the protein-fiber mixture to have a significant impact. Furthermore, a higher rate of protein fermentation was found with a low proportion of WB+PS, possibly due to its starch component (discussed in detail in 5.1.3 above). Clearly, microbial preference for carbohydrates did not strongly deter bacteria from continuing to ferment the protein available. Instead, the biggest impediment to microbial protein fermentation was likely pH. Evidence is provided by a quick comparison of protein-FOS samples to those of protein-WB+PS samples (Figure 5.2). When FOS was the fiber included, pH and total BCFAs dropped significantly, whereas they remained relatively high in WB+PS fiber inclusion samples[8].

Ammonia production was not largely affected by changing carbohydrate availability. As protein substrate was removed and replaced with fiber, it was reasoned that the concentration of ammonia

[8] The pH effect was discussed in more detail within the discussions for FOS (5.1) and WB+PS (5.1.3).

would proportionally decrease. Ammonia levels did decrease (Figure 4.8), but they did not do so in proportion to reduction of protein as had been expected. Appreciable change in ammonia concentration required removal of more protein than anticipated, requiring a reduction of protein by at least half in the case of FOS and three-quarters for the other tested fibers. This contradicts results from a previous *in vitro* study in which addition of a fermentable carbohydrate (as Lintner's starch) inhibited formation of ammonia regardless of pH (Smith & Macfarlane, 1998). Figure 4.8 shows ammonia concentration was relatively stable between the 100% protein control, 25%, and 50% fiber inclusion levels. Looking at it in reverse, one could postulate that, since addition of protein made little difference between those levels, the rate-limiting step was likely within the ammonia production pathway, the population of bacteria capable of ammonia production, or a microbial tolerance limit for ammonia was reached within the fermentation vessel itself.

Ammonia was inhibited for all tested fiber sources when they made up the majority of the protein-fiber substrate (Figure 4.8). At this level (75% fiber inclusion), there were some distinct differences between the fibers, with Pectin and WB+PS having the highest concentration, followed by Even Mix, and finally FOS with the lowest concentration. In dogs, a FOS supplement lowered ammonia concentration by about 10% regardless of high or low protein diet (Pinna et al., 2016). This is in agreement with our results in which inclusion of FOS had the greatest inhibitory effect overall due to its significantly lower pH and protein fermentation activity. Along with pH, modulation of ammonia production is likely dependent on both carbohydrate and protein source. For example, under osmotic stress, *in vitro* fermentation (using pig inoculum) of varied dietary fibers combined with protein differed in ability to lower ammonia production depending on the carbohydrate source, protein source, and microbial enzymatic accessibility to the protein (Rink, Bauer, Eklund, & Mosenthin, 2012). This concurs with our results which found that, at the 75% inclusion level, Even Mix inclusion significantly reduced ammonia production compared to Pectin or WB+PS fibers at the same level despite similar pH. Since protein accessibility presumably remained constant in our study, it can be inferred that the carbohydrate source and pH were significant factors for ammonia production/suppression when only a small amount of protein was available.

Protein can be butyrogenic. On its own, the 100% protein control produced a large amount of butyrate, greater even than some of the 100% fiber controls (Figure 4.12). This supports previous

findings in which less digestible proteins (i.e., more available) were found to increase butyrate *in vitro* (Poelaert et al., 2017), and high-protein diets in rats and pigs increased cecal butyrate concentrations (Adam et al., 2016; Peng et al., 2017).

In contrast however, several human studies have found high protein consumption to decrease fecal butyrate concentrations (Beaumont et al., 2017; Duncan et al., 2007; Russell et al., 2011). A closer look leads one to reconsider the initially drawn conclusions by the authors. In the Duncan et al. study, two diets, a high-protein medium-carbohydrate (HPMC) and a high-protein low-carbohydrate (HPLC), were tested against a control diet. Results indicated that butyrate decreased with decreased carbohydrate; the control diet with the highest level of dietary fiber had the highest butyrate. However, the control diet also had significantly higher levels of BCFAs and ammonia than the HPLC diet, indicating more protein fermentation occurred in the control diet. This may actually indicate that protein fermentation and not carbohydrate led to the higher butyrate production. Unfortunately, the results were confounded by the fact that a high-protein diet with a dietary fiber level similar to the control was not tested. Furthermore, the HPLC diet had a significantly higher percentage of fat as calories than either the HPMC or control diet, so the influence of fat in the diet on gut fermentation outcomes cannot be discounted. The same issue occurs in a similar study that found high-protein diets to be potentially detrimental to gut health (Russell et al., 2011). Again, HPMC and HPLC diets were compared to a control diet in obese men. In this study, the control diet had less protein fermentation than either HP diet and more butyrate than the HPLC diet. Despite the fact that the HPMC diet had nearly 40% less dietary fiber than the control diet, the butyrate concentration between the diets was not significantly different, which may suggest some of the butyrate produced from the HPMC diet was a product of protein fermentation. However, as in the Duncan et al. (2007) study, the HPLC diet had significantly more fat as calories than either the HPMC or control diet, which further confound the results and makes comparisons between the HP diets difficult.

Recalling that the average American gut receives an approximately even amount of protein and dietary fiber (Gaudichon et al., 2002; U.S. Department of Agriculture, Agricultural Research Service, 2018), which coincides with the 50% fiber inclusion levels in our study, most tested fiber sources did not greatly influence butyrate production which remained relatively high regardless of

fiber proportions (Figure 4.12). This is with the exception of Pectin inclusion, which led to decreased butyrate concentration, and corroborates a study in which the addition of pectin reduced butyrate levels in rats (Adam et al., 2016). Compared to the 100% fiber controls, the presence of protein either reduced butyrate (FOS, WB+PS) or increased butyrate (Pectin, Even Mix). However, a protein-fiber mixture more closely represents the colonic environment *in vivo* since protein is available and fermented along the entire length of the colon (Cummings et al., 1987). Therefore, butyrate production values by the 100% fiber controls is likely unrealistic *in vivo*. This may offer some explanation of why seemingly butyrogenic fibers *in vitro* can have inconsistent results *in vivo*.

Protein promoted butyrate-producing bacteria, but when combined with dietary fiber, the fiber source greatly influenced butyrogenic potential (Figure 5.1). Unsurprisingly, the molar proportion of butyrate dropped when protein was combined with fibers that were less butyrogenic (Pectin and Even Mix) and increased when combined with a fiber (WB+PS) that was more butyrogenic. However, in combination with a similarly butyrogenic fiber (FOS) an antagonistic effect occurred, wherein molar butyrate proportion significantly decreased in protein-FOS mixtures compared to their individual controls (100% protein control and 100% FOS fiber control). This was however most probably a consequence of FOS's suppression effect on protein fermentation and not truly a reduction in FOS's ability to promote butyrate-producing bacteria.

The potential of protein to produce butyrate and promote butyrate-producing bacteria is intriguing since one of the current goals in the microbiome field is increasing butyrate in the distal colon. Furthermore, protein fermentation produces less gas, and accordingly, in our study, protein quantity was negatively correlated with gas production ($r = -0.8805$, $p < 0.0001$). The implication is that butyrate-producing bacteria could be promoted by protein fermentation while also having the additional benefit of less pain or discomfort from excessive gas production in the colon. It has, however, been suggested butyrate produced via protein fermentation may not be as beneficial because of the concurrent production of ammonia (Diether & Willing, 2019). Ammonia is known to decrease butyrate uptake by colonocytes, decrease colonocyte oxidation of butyrate, and have the overall effect of disrupting epithelial cell integrity and barrier function (Anand, Kaur, & Mande, 2016; Blachier et al., 2007; Darcy-Vrillon et al., 1996; Villodre Tudela et al., 2015). Additionally,

the notion of increasing butyrate with protein must be balanced with the potentially severe consequences of protein fermentation in the colon. Numerous studies have linked colonic protein fermentation and/or its metabolites with carcinoma, ulcerative colitis, chronic bowel inflammation, colonic DNA damage, and promotion of pathogenic bacteria (Armstrong & Doll, 1975; Christl, Eisner, Dusel, Kasper, & Scheppach, W., 1996; Le Leu et al., 2006; Levine et al., 2014; Toden, Bird, Topping, & Conlon, 2006; Toden et al., 2007). Separately, studies have also linked increased red meat consumption with colorectal cancer (Armstrong & Doll, 1975; Bingham et al., 1996; Giovannucci et al., 1994; Wada et al., 2017; World Cancer Research Fund/American Institute for Cancer Research, 2018a). The World Health Organization (WHO) declared consumption of red meat as probably carcinogenic and processed meats as carcinogenic to humans (International Agency for Research on Cancer, 2015). Nevertheless, the research on factors affecting protein fermentation such as dietary source and processing, as well as gut and health outcomes specific to these factors, is still in its infancy and is covered in several recent reviews (Blachier et al., 2019; Davila et al., 2013; Diether & Willing, 2019; Portune et al., 2016; Yao et al., 2016).

5.3 Study Limitations and Future Work

One limitation of the study was using a batch culture *in vitro* fermentation assay, which does not precisely mimic the colonic pH. In the colonic environment, many metabolites are capable of being absorbed through the epithelial or used by the intestinal epithelial cells. However, *in vitro*, there is accumulation of microbial byproducts, such as SCFAs and ammonia, causing the pH to be artificially influenced. The pH affects the promotion or suppression of microbiota, and subsequently, the very things this study sought to measure: microbial protein fermentation and metabolites. Therefore, future studies could better mimic the human colonic environment by utilizing an *in vitro* fermentation assay that removes metabolites or through the use of an animal model.

Compared to the plethora of research conducted on carbohydrate fermentation in the gut, there are relatively few studies on colonic protein fermentation. Even smaller are the number of studies focused on the influence of carbohydrates on gut microbial protein fermentation and vice versa. Therefore, a goal of this study was to gain broad, foundational knowledge to lay the groundwork for further studies. This, however, brings with it some limitations, most notably in the microbiota

present and the substrates presented to those microbiota. The molecular structure and complexity of proteins and carbohydrates vary greatly by source and may be altered by food processing and cooking. This leads to variance in digestibility and therefore the amount, or even the form (e.g., polypeptides versus proteins), that arrives in the colon available for microbial fermentation. Therefore, the use of a single protein mixture and four fibers does not mimic the variability found in the human diet.

The ecology of the microbiome is unique to each individual. With this in mind, fecal sample donations were pooled; this method was used with the idea that results would be applicable to the general population rather than a specific individual. The downside to this approach, however, is that avoidance of individual specificity results in data specific to no one.

Further, the particular changes to the ecological community of the microbiota during microbiome studies provides additional insight and understanding. For example, a pattern of microbial shifts might be correlated to specific substrates and/or individuals in certain disease states. Additionally, it is important to confirm if commensal or pathogenic bacteria are responsible for positive or negative metabolite changes.

First and foremost, future work should focus on determining microbial shifts associated with the metabolites produced during the fermentation of the protein and fibers used in this study. This is particularly important because this thesis study showed that fermentation of protein can be butyrogenic, which is considered to be a highly beneficial outcome. However, it is paramount to confirm the butyrogenic effect was not due to, or concomitant with, promotion of pathogenic bacteria. Further studies should also address how different proteins, fibers, and their processing affect the level of influence each has on fermentation profiles and metabolites produced. Finally, future studies may also consider using separate, and not pooled, fecal samples from many individuals. This may result in determining how an individual's normal diet habits and initial microbial ecology affect overall outcomes. Additionally, the presence of potentially universal outcomes might be identified.

5.4 Closing Remarks

The usefulness of dietary fiber to effectively inhibit protein fermentation in the gut remains unclear. Furthermore, protein may be butyrogenic and therefore could have some positive influence on gut health. Still, this must be tempered with increased levels of ammonia and other potentially toxic metabolites as well as the associations between animal protein intake levels and colorectal cancer. Based on this study, high dietary fiber diets with a moderate level of protein intake as recommended by governmental health agencies, would result in lower ammonia concentrations, even if the overall protein fermentation activity is unchanged. Furthermore, this type of diet would be beneficial due to lowering pH, increasing SCFAs, and in most cases having similar levels of butyrate production compared to high-protein low-dietary fiber diets. More research is needed in this area, particularly due to the increasing popularity of high-protein low-carbohydrate diets for weight loss. It is likely that due to its ubiquity in the human gut, protein fermentation could have beneficial effects. Future work should focus on understanding the specific influences of dietary proteins and fibers on shaping the gut microbiome. This knowledge could lead to improved dietary recommendations or even recommendations tailored to the individual in order to improve or maintain health.

Table 5.1. Mean SCFA ratio of acetate, propionate, and butyrate after fermentation of protein-fiber substrate mixtures for 24 and 48 hours (n=3). 'Fiber Source' = the fiber(s) used in the protein-fiber mixture, 'Fiber Inclusion' = the proportion of fiber in the protein-fiber mixture, 'Control' = 100% protein, 0% fiber, 'Fiber Control' = 0% protein, 100% fiber, 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

Fiber Source	*Fiber Inclusion*	**Acetate : Propionate : Butyrate**	
		24 hrs	*48 hrs*
Control	N/A	61:24:14	62:25:14
FOS	25%	67:21:12	68:21:11
	50%	71:19:11	72:18:10
	75%	73:19:09	74:18:09
	Fiber Control	68:20:12	71:18:11
Pectin	25%	67:22:11	66:22:11
	50%	73:19:09	72:19:09
	75%	78:15:07	80:14:06
	Fiber Control	84:12:05	83:12:05
WB+PS	25%	62:24:14	63:24:13
	50%	64:22:14	64:22:14
	75%	67:19:14	66:20:14
	Fiber Control	69:16:15	68:16:15
Even Mix	25%	66:21:12	66:22:12
	50%	73:18:10	71:18:10
	75%	78:14:08	76:15:09
	Fiber Control	82:11:07	80:12:08

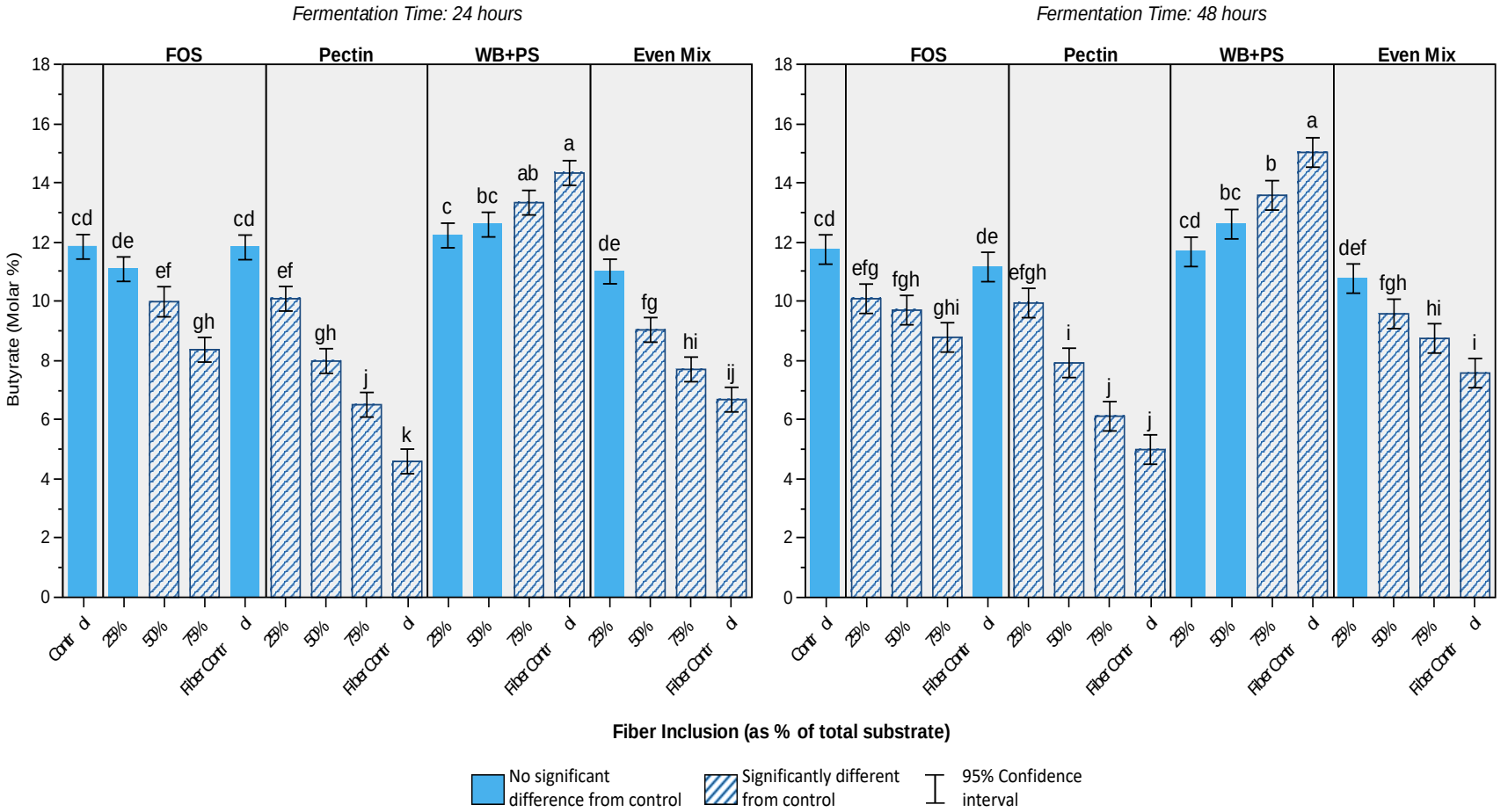

Figure 5.1. Least square mean molar proportion of butyrate (as percent of total short- and branched-chain fatty acids) after 24 and 48 hours fermentation of protein-fiber substrate mixtures (n=3), grouped by fermentation time and fiber substrate source (top). X-axis represents the percentage of fiber in the protein-fiber fermentation substrate mixture. 'Blank' = no substrate, inoculum only, 'Control' = 100% protein, 0% fiber substrate, 'Fiber Control' = 0% protein, 100% fiber. Within each time point, bars with a common letter are not significantly different by Tukey's HSD-test at the 5% level of significance. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

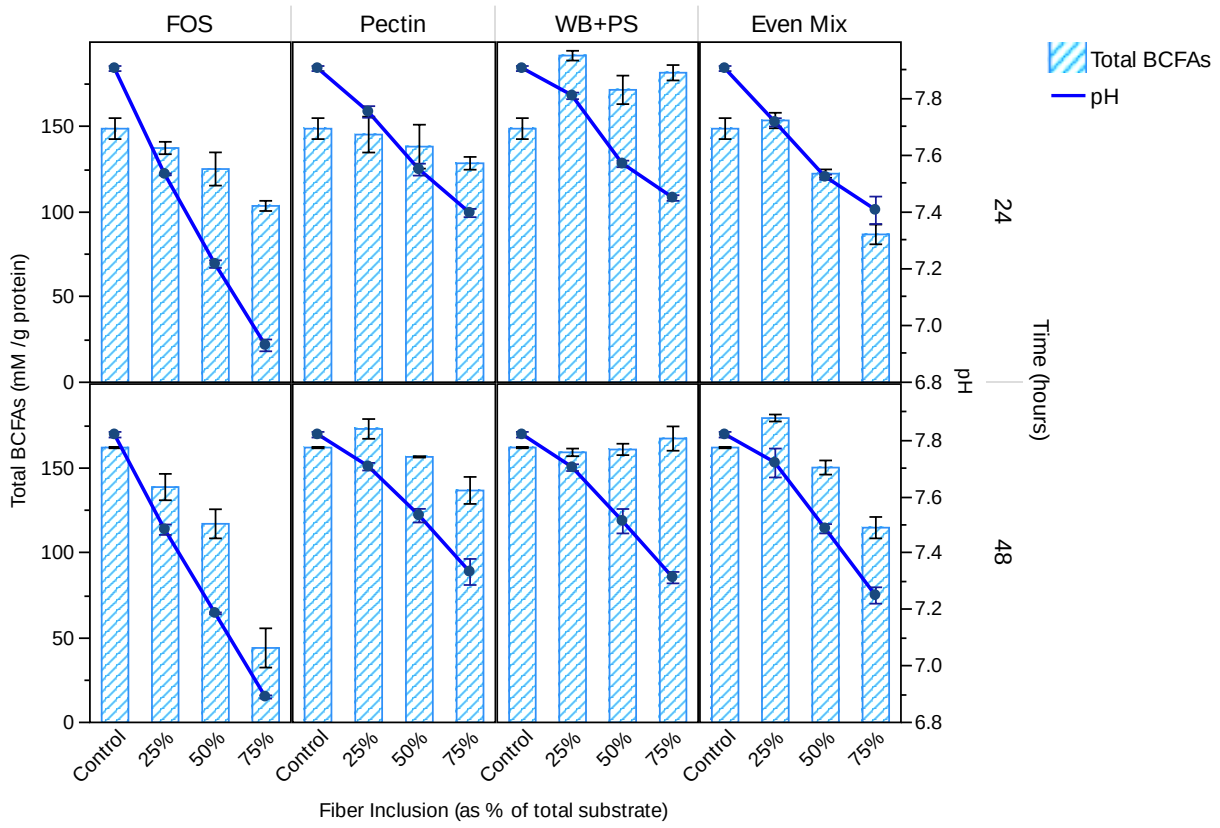

Figure 5.2. Mean total BCFA (mM/g protein basis) (bars) and mean pH (connected points, secondary y-axis) after fermentation of protein-fiber substrate mixtures for 24 and 48 hours (n=3). Total BCFAs is the sum of iso-butyrate and iso-valerate. Fiber sources (top labels) represent the fiber used as part of the protein-fiber mixture; X-axis represents the proportion of fiber in the protein-fiber mixture; 'Control' = 100% protein, 0% fiber. The Control is repeated in the figure within each fiber source for easier comparison. Error bars represent standard error. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

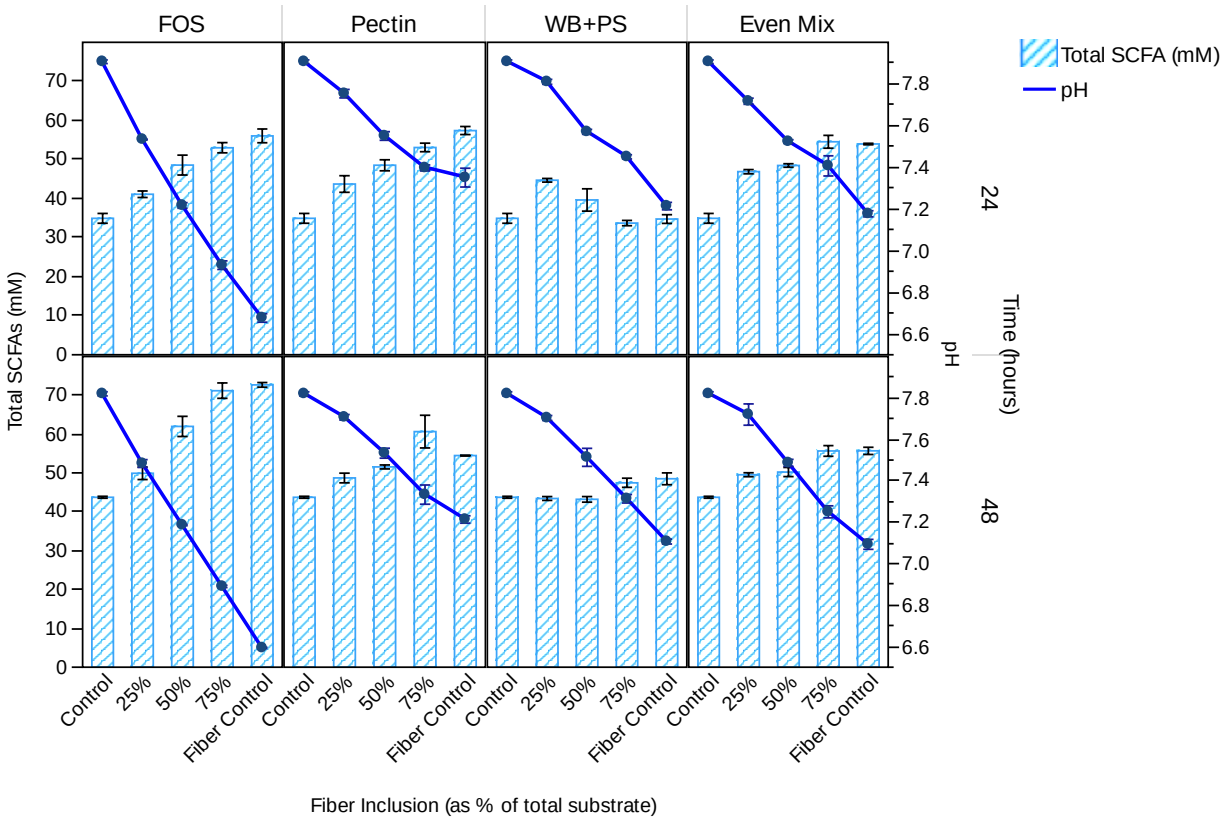

Figure 5.3. Mean total SCFAs (bars) and mean pH (connected points, secondary y-axis) after fermentation of protein-fiber substrate mixtures for 24 and 48 hours (n=3). Total SCFAs is the sum of acetate, propionate, and butyrate. Fiber sources (top labels) represent the fiber used as part of the protein-fiber mixture; X-axis represents the proportion of fiber in the protein-fiber mixture; 'Control' = 100% protein, 0% fiber; 'Fiber Control' = 0% protein, 100% fiber. The Control is repeated in the figure within each fiber source for easier comparison. Error bars represent standard error. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

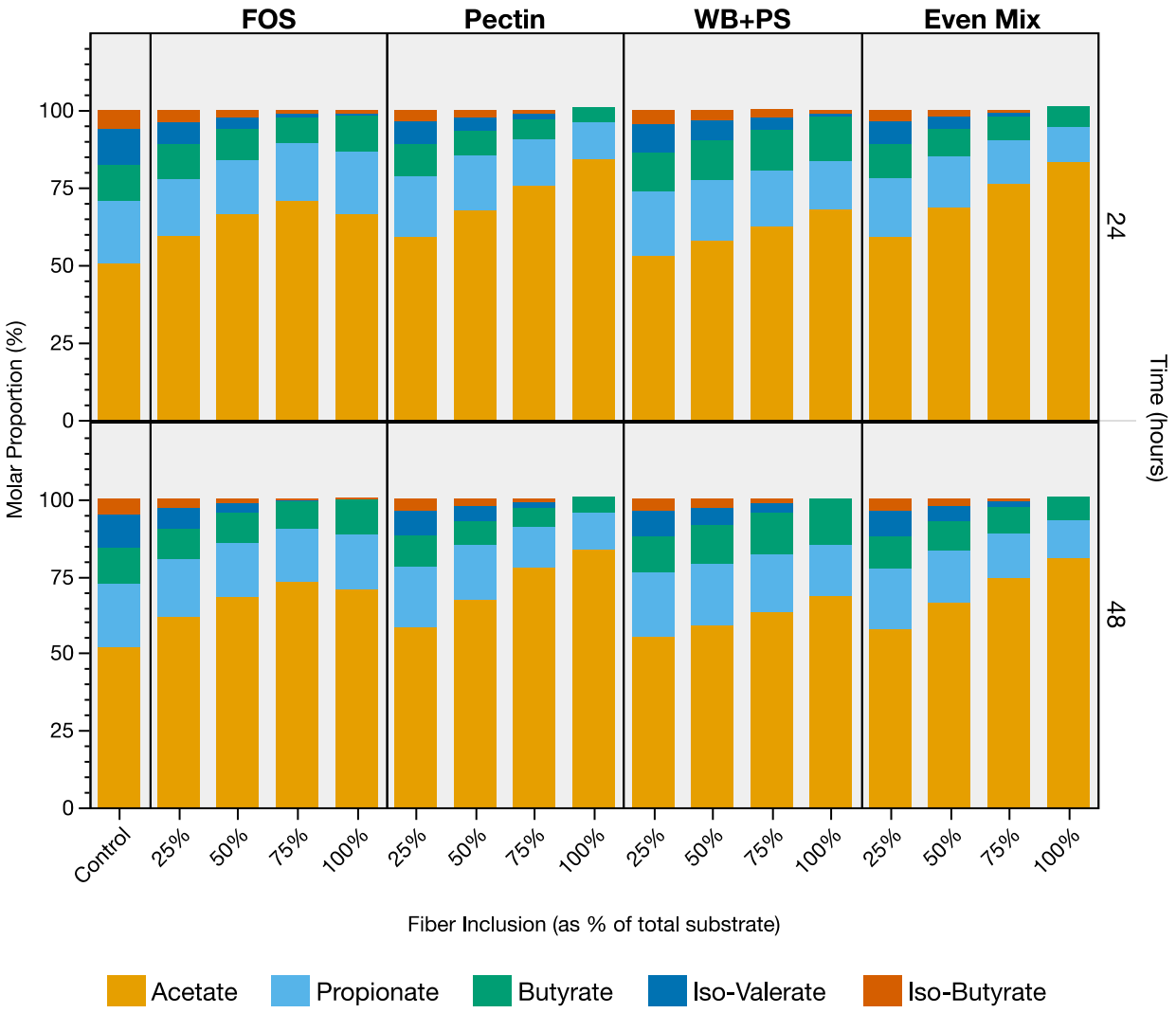

Figure 5.4. Mean molar proportion of acetate, propionate, butyrate, iso-valerate, and iso-butyrate of total short-chain and branched-chain fatty acids after fermentation of protein-fiber substrate mixtures for 24 and 48 hours (n=3). Fiber sources (top labels) represent the fiber used as part of the protein-fiber mixture; X-axis represents the proportion of fiber in the protein-fiber mixture; 'Control' = 100% protein, 0% fiber. 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

APPENDIX

Table A.1. BCFA concentrations (mM) of protein-fiber mixtures after 0, 24, and 48 hours fermentation. Values are expressed as means ± standard deviation (n=3). Total BCFAs is the sum of iso-butyrate and iso-valerate. 'Fiber Source' = the fiber(s) used in the protein-fiber mixture, 'Fiber Inclusion' = the proportion of fiber in the protein-fiber mixture, 'Blank' = no substrate, inoculum only, 'Control' = 100% protein, 0% fiber, 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

	Fiber	**Iso-Butyrate (mM)**			**Iso-Valerate (mM)**			**Total BCFAs (mM)**		
Fiber Source	*Inclusion*	*0 hrs*	*24 hrs*	*48 hrs*	*0 hrs*	*24 hrs*	*48 hrs*	*0 hrs*	*24 hrs*	*48 hrs*
Blank	N/A	0.18 ± 0.01	0.20 ± 0.01	-0.35 ± 0.01	0.21 ± 0.01	0.24 ± 0.01	-0.38 ± 0.02	0.38 ± 0.01	0.45 ± 0.01	-0.73 ± 0.03
Control	N/A		2.51 ± 0.18	2.61 ± 0.01		4.95 ± 0.35	5.51 ± 0.04		7.46 ± 0.53	8.12 ± 0.05
FOS	25%		1.78 ± 0.09	1.66 ± 0.17		3.38 ± 0.15	3.56 ± 0.34		5.16 ± 0.24	5.22 ± 0.51
	50%		1.14 ± 0.13	0.94 ± 0.13		2.01 ± 0.21	2.00 ± 0.25		2.10 ± 1.83	2.94 ± 0.38
	75%		0.65 ± 0.03	0.30 ± 0.08		0.64 ± 0.03	0.25 ± 0.18		1.30 ± 0.06	0.55 ± 0.25
	100%		0.60 ± 0.01	0.28 ± 0.01		0.39 ± 0.01	-0.13 ± 0.01		0.99 ± 0.02	0.15 ± 0.02
Pectin	25%		1.74 ± 0.23	2.08 ± 0.12		3.72 ± 0.46	4.44 ± 0.25		5.46 ± 0.69	6.52 ± 0.37
	50%		1.14 ± 0.19	1.31 ± 0.01		2.34 ± 0.36	2.62 ± 0.01		3.48 ± 0.55	3.92 ± 0.02
	75%		0.63 ± 0.03	0.67 ± 0.08		0.98 ± 0.06	1.05 ± 0.10		1.61 ± 0.09	1.72 ± 0.18
	100%		-0.16 ± 0.01	-0.10 ± 0.01		-0.29 ± 0.01	-0.18 ± 0.01		-0.45 ± 0.02	-0.28 ± 0.02
WB+PS	25%		2.31 ± 0.06	1.93 ± 0.05		4.91 ± 0.13	4.05 ± 0.08		7.21 ± 0.18	5.98 ± 0.13
	50%		1.45 ± 0.06	1.32 ± 0.05		2.86 ± 0.32	2.72 ± 0.10		4.31 ± 0.37	4.04 ± 0.15
	75%		0.90 ± 0.03	0.72 ± 0.06		1.39 ± 0.05	1.39 ± 0.09		2.28 ± 0.08	2.11 ± 0.15
	100%		0.37 ± 0.01	0.01 ± 0.02		0.38 ± 0.01	0.00 ± 0.02		0.76 ± 0.01	0.02 ± 0.04
Even Mix	25%		1.84 ± 0.10	2.15 ± 0.04		3.93 ± 0.21	4.60 ± 0.11		5.77 ± 0.31	6.75 ± 0.15
	50%		0.98 ± 0.04	1.22 ± 0.06		2.09 ± 0.06	2.55 ± 0.11		3.07 ± 0.10	3.77 ± 0.17
	75%		0.39 ± 0.05	0.52 ± 0.07		0.70 ± 0.07	0.93 ± 0.09		1.09 ± 0.13	1.45 ± 0.15
	100%		-0.25 ± 0.01	-0.15 ± 0.01		-0.35 ± 0.02	-0.19 ± 0.02		-0.60 ± 0.02	-0.34 ± 0.03

Table A.2. SCFA concentrations (mM) of protein-fiber mixtures after 0, 24, and 48 hours fermentation. Values are expressed as means ± standard deviation (n=3). Total SCFAs = the sum of acetate, propionate, and butyrate. 'Fiber Source' = the fiber(s) used in the protein-fiber mixture, 'Fiber Inclusion' = the proportion of fiber in the protein-fiber mixture, 'Blank' = no substrate, inoculum only, 'Control' = 100% protein, 0% fiber, 'FOS' = fructooligosaccharides, 'Pectin' = apple pectin, 'WB+PS' = wheat bran and raw potato starch mixture, 'Even Mix' = an even mixture of FOS, Pectin, and WB+PS.

		Acetate (mM)			Propionate (mM)			Butyrate (mM)			Total SCFA (mM)		
Fiber Source	*Fiber Inclusion*	*0 hrs*	*24 hrs*	*48 hrs*	*0 hrs*	*24 hrs*	*48 hrs*	*0 hrs*	*24 hrs*	*48 hrs*	*0 hrs*	*24 hrs*	*48 hrs*
Blank	N/A	2.22 ± 0.01	3.20 ± 0.11	1.10 ± 0.17	0.49 ± 0.01	0.65 ± 0.02	-0.13 ± 0.05	0.48 ± 0.01	0.61 ± 0.02	-0.42 ± 0.03	3.18 ± 0.03	4.46 ± 0.15	0.54 ± 0.24
Control	N/A		21.38 ± 1.43	26.91 ± 0.28		8.51 ± 0.57	10.75 ± 0.11		5.01 ± 0.29	6.09 ± 0.11		34.90 ± 2.17	43.75 ± 0.44
FOS	25%		27.43 ± 0.92	34.03 ± 1.54		8.55 ± 0.35	10.34 ± 0.72		5.13 ± 0.19	5.57 ± 0.52		41.10 ± 1.45	49.94 ± 2.77
	50%		34.26 ± 2.41	44.38 ± 2.97		9.13 ± 0.71	11.32 ± 0.94		5.17 ± 0.49	6.31 ± 0.62		48.55 ± 3.61	62.01 ± 4.51
	75%		38.49 ± 1.57	52.38 ± 2.22		9.97 ± 0.52	12.52 ± 0.76		4.54 ± 0.17	6.31 ± 0.78		53.00 ± 2.25	71.21 ± 3.45
	100%		37.86 ± 2.11	51.45 ± 0.81		11.43 ± 0.62	13.13 ± 0.28		6.74 ± 0.34	8.13 ± 0.34		56.02 ± 3.06	72.72 ± 1.02
Pectin	25%		29.10 ± 2.16	32.31 ± 1.39		9.64 ± 0.95	10.93 ± 0.52		4.97 ± 0.59	5.49 ± 0.31		43.70 ± 3.67	48.73 ± 2.17
	50%		35.18 ± 1.33	37.34 ± 0.71		9.17 ± 0.69	9.83 ± 0.14		4.16 ± 0.58	4.39 ± 0.02		48.51 ± 2.45	51.57 ± 0.86
	75%		41.39 ± 1.59	48.56 ± 7.42		8.12 ± 0.21	8.31 ± 0.34		3.56 ± 0.12	3.80 ± 0.20		53.07 ± 1.90	60.66 ± 7.30
	100%		47.97 ± 1.56	45.31 ± 0.17		6.81 ± 0.21	6.47 ± 0.02		2.61 ± 0.04	2.71 ± 0.06		57.39 ± 1.79	54.49 ± 0.20
WB+PS	25%		27.52 ± 0.50	27.38 ± 0.48		10.80 ± 0.13	10.30 ± 0.29		6.34 ± 0.22	5.77 ± 0.06		44.66 ± 0.84	43.45 ± 0.83
	50%		25.40 ± 2.99	27.85 ± 0.69		8.70 ± 1.29	9.41 ± 0.33		5.53 ± 0.68	5.96 ± 0.27		39.63 ± 4.95	43.22 ± 1.24
	75%		22.48 ± 0.71	31.33 ± 1.19		6.43 ± 0.29	9.42 ± 0.44		4.80 ± 0.20	6.74 ± 0.40		33.70 ± 1.19	47.49 ± 2.02
	100%		24.14 ± 1.35	33.22 ± 1.52		5.51 ± 0.32	7.99 ± 0.23		5.09 ± 0.26	7.31 ± 1.03		34.74 ± 1.92	48.53 ± 2.64
Even Mix	25%		31.15 ± 0.54	32.59 ± 0.73		9.92 ± 0.24	10.91 ± 0.15		5.79 ± 0.21	6.06 ± 0.05		46.86 ± 0.96	49.57 ± 0.86
	50%		35.30 ± 0.56	35.85 ± 1.43		8.49 ± 0.19	9.24 ± 0.39		4.65 ± 0.07	5.17 ± 0.23		48.45 ± 0.79	50.26 ± 2.04
	75%		42.42 ± 2.15	42.45 ± 1.72		7.81 ± 0.53	8.24 ± 0.34		4.28 ± 0.31	5.00 ± 0.34		54.52 ± 2.84	55.69 ± 2.37
	100%		44.40 ± 0.22	44.84 ± 1.54		5.98 ± 0.01	6.69 ± 0.19		3.56 ± 0.16	4.19 ± 0.14		53.94 ± 0.33	55.71 ± 1.59

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