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Identification of Gut Microbiome Composition Responsible for Gas Production

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IDENTIFICATION OF GUT MICROBIOME COMPOSITION RESPONSIBLE FOR GAS PRODUCTION

by

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Gas production remains a significant barrier to increasing intake of dietary fiber-containing foods for many consumers. Therefore, this thesis is comprised of two studies focusing on the role of the gut microbiome in contributing to gas production during fermentation of dietary components. Previous studies have reported significant correlations between *Megasphaera elsdenii* and gas production during fermentation of pulses. Therefore, the objective of the first study was to determine the role of *M. elsdenii* in gas production by the microbiome during fermentation of foods commonly associated with gas production. Human fecal microbiomes were separated based on the presence (Me+) or absence (Me–) of *M. elsdenii*. After 48 h of fermentation, Me+ microbiomes produced significantly more gas than the Me− microbiomes. Furthermore, Me+ microbiomes were more butyrogenic than Me– microbiomes, while Me– microbiomes were more acetogenic and propiogenic. This study suggested that *M. elsdenii* may be responsible for high gas production during consumption of flatulogenic foods. In the second study, raffinose, an oligosaccharide found in pulses and implicated in gas-production, was used as a substrate for *in vitro* fermentation. The objective of this study was to determine the relationship between raffinose utilization and gas production and identify microbial features that were responsible for gas production during fermentation

of raffinose. Unexpectedly, raffinose utilization was negatively correlated with gas production. Raffinose utilization was also positively correlated with acetate production, while gas production was positively correlated with butyrate production. Taxa from *Bifidobacterium* and *Blautia* were associated with raffinose degradation and acetate production. Several taxa from *Megasphaera, Anaerostipes, Faecalibacterium,* and *Collinsela,* were associated with gas and butyrate production. This study suggested that gas production was not produced directly from the metabolism of raffinose, but rather through cross-feeding between raffinose-degrading, acetate-producing bacteria and acetate-utilizing, butyrate-producing bacteria. Overall, this research has revealed substantial variation in gas production among microbiomes and identified commensal members of the microbiome and cross-feeding pathways that contribute to elevated gas production by the microbiome. These findings will be important in the development of strategies to reduce undesirable gas production during consumption of flatulogenic foods.

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CHAPTER 1 INTESTINAL GAS PRODUCTION BY THE GUT MICROBIOTA: A REVIEW

ABSTRACT

In addition to causing embarrassment, intestinal gas can be associated with more serious symptoms. This review provides an overview of gas production by the human gut microbiome and outlines foods associated with intestinal gas. *Bacteroides*, *Ruminococcus*, *Roseburia*, *Clostridium*, *Eubacterium*, *Desulfovibrio,* and *Methanobrevibacter* are among the most abundant microbes responsible for intestinal gas. More than 99% of intestinal gas is composed of hydrogen, carbon dioxide, and methane, while less than 1% is composed of other odiferous gases. Food groups associated with intestinal gas include pulses, vegetables, fruits, grains, and, for some individuals, dairy. These foods are rich in non–digestible carbohydrates such as raffinose family oligosaccharides, fructans, polyols, and, for sensitive individuals, lactose. These carbohydrates are fermented by colonic bacteria and produce gases directly or by cross feeding. Additional research on gas production by the gut microbiota and foods associated with gas may help mitigate the symptoms linked to intestinal gas.

1.1 Introduction

During typical metabolism of dietary and endogenous components in the large intestine, the gut microbiota of most healthy people can generate 0.2L–1.5L of gas per day (Mego, Accarino, Malagelada, Guarner, & Azpiroz, 2015; Serra, Azpiroz, & Malagelada, 1998; Suarez, Springfield, & Levitt, 1998). The gases predominantly produced by the gut microbiome include hydrogen (H_2), carbon dioxide (CO_2), and methane (CH_4). These gases contribute to more than 99% of the intestinal gas volume and are odorless (Suarez & Levitt, 2000). The unpleasant odor associated with intestinal gas comprises less than 1% of intestine gas volume and is the result of the sulfur–containing trace gases such as hydrogen sulfide (H_2S), methanethiol (CH_3SH), and dimethyl sulfide [$(CH_3)_2S$] (Kalantar-Zadeh, Berean, Burgell, Muir, & Gibson, 2019; Suarez et al., 1998). Microbiota–generated gas is expelled from the digestive tract through the mouth by belching, through the lungs after diffusion into the blood, and through the anus as flatus.

In general, besides being socially awkward or causing embarrassment in public circumstances, intestinal gas is normal for most healthy people (Tomlin, Lowis, & Read, 1991). However, intestinal gas is reported to be associated with other abdominal symptoms such as bloating, constipation, belching, abdominal pain, and excessive passing of gas. Excessive intestinal gas can have a negative impact on the social well–being of an individual and may also be a symptom of chronic conditions such as irritable bowel syndrome (Caldarella, Serra, Azpiroz, & Malagelada, 2002). Indeed, intestinal gas is one of the most common health complaints that makes people visit a gastroenterologist (Azpiroz & Michael, 2010)(Manichanh et al., 2014a).

Unfortunately, most of the foods that promote human health, including vegetables, high–fiber grains, and legumes, are known to contribute to intestinal gas (Manichanh et al., 2014b; Mego et al., 2015; Winham, Webb, & Barr, 2008). These foods have unabsorbed residues that can be fermented in the colon by gut bacteria and lead to gas production as a by–product of microbial metabolism (Mego et al., 2015). The purpose of this review is to provide an overview of the gas–producing pathways used by the human gut microbiome and to outline foods associated with intestinal gas.

1.2 Gut microbiota, gases, and disease

The human gut is host to trillions of bacteria largely composed of strictly anaerobic microorganisms. The overall microbial population in the gastrointestinal tract, also known as the gut microbiota, varies widely from one person to another based on different factors such as diet, host genetics, and environmental conditions (Holscher, 2017). Although microbes that colonize the intestinal lumen may vary widely between individuals, the majority of bacterial species belong to five phyla, which are Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia (Rinninella et al., 2019). These microorganisms contribute significantly to the health and disease of the host (Tremaroli & Bäckhed, 2012).

Among other functions, bacteria in the colon have the ability to ferment the substrates that are not digested or absorbed in the upper gastrointestinal tract. These include carbohydrates, proteins, fats, and other dietary components that escape digestion due to molecular or physical structural complexity (Yao, Muir, & Gibson, 2016). Primary fermenters break these nutrients down for energy and metabolism and release metabolites such as short chain fatty acids (e.g., acetate, propionate, and butyrate) and various gases

as by–products or intermediates of fermentation (Bernalier-Donadille, 2010). Human gut microorganisms form complex microbial communities that depend on one another to harvest nutrients and energy to survive (Pimentel, Mathur, & Chang, 2013); in fact, metabolites produced by one strain in the community may be further utilized by another (Smith, Shorten, Altermann, Roy, & McNabb, 2019). The metabolites released by microbiotas differ across individuals (Rinninella et al., 2019; Smith et al., 2019). Although some microbial fermentation products, especially short chain fatty acids, impact human health and are beneficial to the host, gases produced during fermentation can be detrimental to many people.

Although the majority of species belong to five phyla, the two major bacterial phyla responsible for gas production in the gut belong to Bacteroidetes and Firmicutes and together they make up over 90% of the total bacterial population of the human gut (Arumugam et al., 2013). Bacteria in these phyla produce primarily H_2 and CO_2 (Hylemon, Harris, & Ridlon, 2018; Kalantar-Zadeh et al., 2019). CH_4 gas is also produced by colonic microbes from the metabolism of CO_2 and H_2 by Archaea in the colon. Minor gases such as H_2S and other sulfur–containing gases are produced in trace concentrations by sulfur–reducing bacteria (SRB), which can reduce sulfate compounds to H_2S (F. Suarez, Furne, Springfield, & Levitt, 1997). Sulfate may be derived in the colon from sources such as proteins from animal foods which contain amino acids cysteine, methionine, and taurine, as well as carrageenan and other sulfated polysaccharides (Rey et al., 2013).

1.2.1 Hydrogen

H_2 is the predominant gas produced by colonic bacteria and is produced solely through bacterial fermentation of non–digestible substrates in the colon (Naito, Uchiyama, & Takagi, 2018). Accordingly, breath H_2 has been used as a primary marker for diagnostic testing of carbohydrate malabsorption or small intestinal bacterial overgrowth (SIBO). Fermentation by the gut bacteria creates the potential for large quantities of H_2 gas to be produced within the gut. It has been reported that up to 1L of H_2 can be produced in 24 h, and this magnitude of gas can cause gastrointestinal symptoms such as bloating, abdominal pain, and excessive flatus (Gasbarrini et al., 2009; Strocchi & Levitt, 1992).

The most abundant bacterial genera responsible for H_2 production in the colon are *Bacteroides*, *Ruminococcus*, and *Roseburia* (Duncan, Hold, Barcenilla, Stewart, & Flint, 2002; Zheng, Kahnt, Kwon, Mackie, & Thauer, 2014). Other colonic taxa known to be associated with H_2 production include *Anaerostipes caccae*, *Clostridium* spp., *Eubacterium rectale*, *Enterococcus*, and *Victivallis vadensis* (Table 1.1) (Duncan & Flint, 2008; Ivan Kushkevych, 2013; Schwiertz et al., 2002; Steer, Collins, Gibson, Hippe, & Lawson, 2001; Zoetendal, Plugge, Akkermans, & de Vos, 2003).

Table 1.1. Major gas producing microbes present in the human gut microbiome

Phylum	Genus and/or species	Gas	Reference
Bacteroidetes	*Parabacteroides*	H_2 & CO_2	(Ezeji et al., 2021)
	Alistipes	H_2 & CO_2	(Oliphant & Allen-Vercoe, 2019)
	Bacteroides	H_2 & CO_2	(Smith et al., 2019)
	Enterococcus	H_2 & CO_2	(Robert & Bernalier-Donadille, 2003)
Firmicutes			
	Dorea	H_2 & CO_2	(Oliphant & Allen-Vercoe, 2019)
	Clostridium spp.	H_2 & CO_2	(Steer et al., 2001)
	Roseburia intestinalis	H_2 & CO_2	(Duncan et al., 2002)
	Ruminococcus	H_2 & CO_2	(Zheng et al., 2014)
	Anaerostipes caccae	H_2 & CO_2	(Schwiertz et al., 2002)
	Eubacterium rectale	H_2 & CO_2	(Duncan & Flint, 2008)
	Blautia	H_2 & CO_2	(Suzuki et al., 2018)
	Veillonella	H_2 & CO_2	(Aujoulat, Bouvet, Jumas-Bilak, Jean-Pierre, & Marchandin, 2014)
	Victivallis vadensis	H_2 & CO_2	(Zoetendal et al., 2003)
	Desulfotomaculum	H_2S	(Dordević, Jančíková, Vítězová, & Kushkevych, 2021)
Proteobacteria	*Desulfovibrio piger*	H_2S	(Rey et al., 2013)
	Desulfovibrio fairfieldensis	H_2S	(Ivan Kushkevych, Dordević, Kollar, Vítězová, & Drago, 2019)
	Desulfovibrio desulfuricans	H_2S	(Ivan Kushkevych, 2016)
	Desulfobulbus	H_2S	(Dordević et al., 2021)
	Desulfomicrobium	H_2S	(Ivan Kushkevych, 2014)
	Desulfomonas	H_2S	(Ivan Kushkevych, 2014)
	Fusobacterium spp.	H_2S	(Mothersole & Wolthers, 2019)
	Bilophila	H_2S	(Braccia, Jiang, Pop, & Hall, 2021)
	Escherichia	H_2 & CO_2	(Suzuki et al., 2018)
Euryarchaeota	*Methanobrevibacter smithii*	CH_4	(Weaver, Krause, Miller, & Wolin, 1986)
	Methanosphaera stadtmanae	CH_4	(Fricke et al., 2006)

Methannobrevibacter oralis	CH_4	(Scanlan, Shanahan, & Marchesi, 2008)

The most common pathway used by bacteria to produce H_2 is the Embden–Meyerhof–Parnas pathway, also known as glycolysis. The majority of gut bacteria use this pathway to convert carbohydrates into pyruvate. The oxidation of reduced flavin (FADH) and nicotinamide adenine dinucleotides (NADH) by microbial hydrogenases is the reaction in the Embden–Meyerhof–Parnas pathway responsible for most H_2 produced in the colon (Fig. 1.1) (Den Besten et al., 2013; Hylemon et al., 2018). Other mechanisms by which H_2 can be produced include 1) cleavage of pyruvate to formate and subsequent metabolism by formate hydrogenlyase; 2) generation from pyruvate through the activity of pyruvate: ferredoxin oxidoreductase and hydrogenase (Carbonero, Benefiel, & Gaskins, 2012; Louis, Hold, & Flint, 2014; Macfarlane & Gibson, 1997).

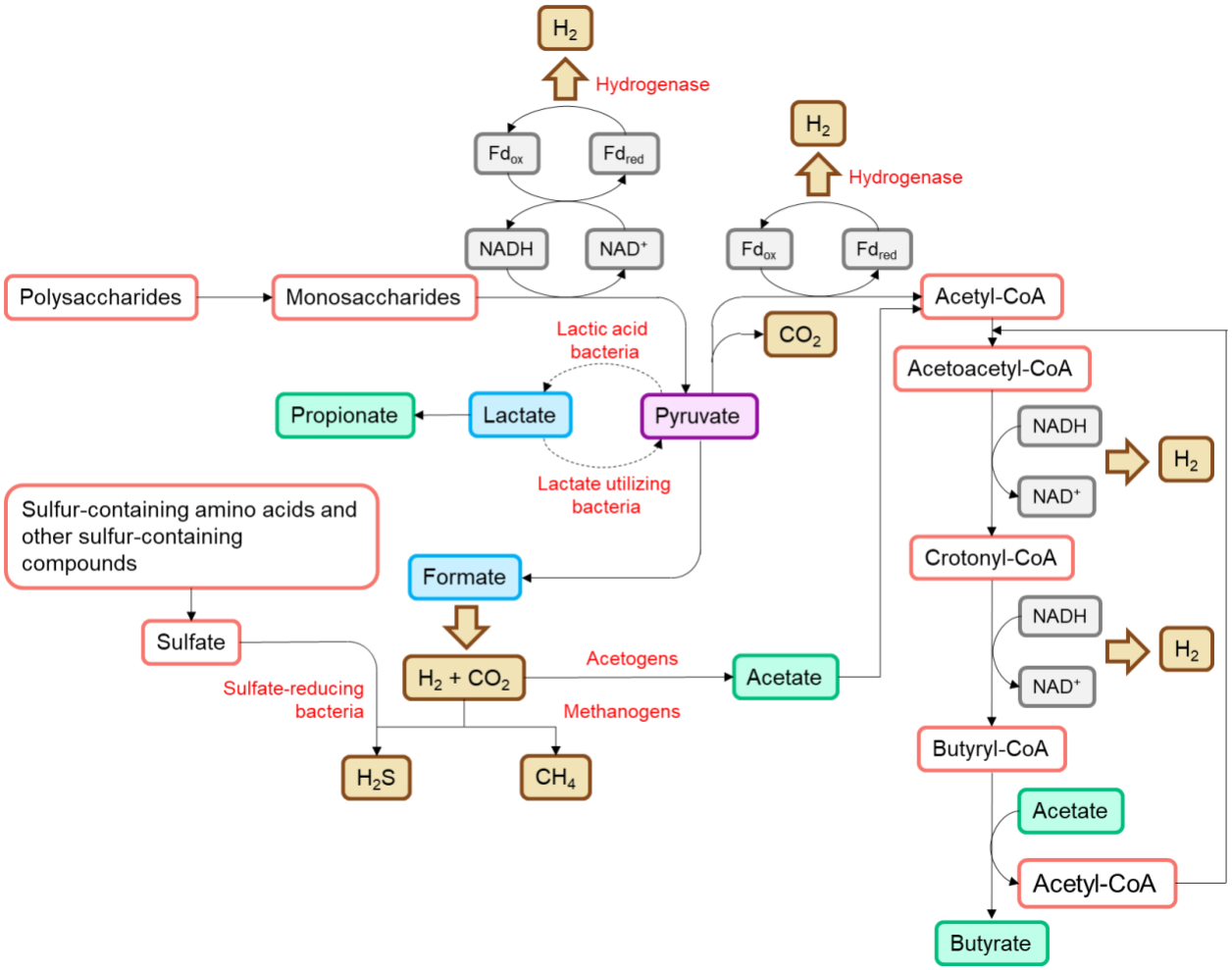

Figure 1.1. Biochemical pathway of H_2, H_2S, CH_4, and CO_2 production from bacterial fermentation. Gases are shown in brown boxes; intermediate products of bacterial fermentation are shown in blue boxes and primary products of bacterial fermentation (short chain fatty acids) are shown in green boxes. Fd_{ox}, oxidized flavin adenine dinucleotide; Fd_{red}, reduced flavin adenine dinucleotide.

The balance of H_2 concentration in the gut is crucial to colonic fermentation and the host. Indeed, high H_2 partial pressure can hinder bacterial fermentation. Therefore, to prevent the accumulation of H_2 in the gut, the removal of excess H_2 can be mediated by both the host and gut microbiota (Carbonero et al., 2012). About one–third of H_2 produced in the gut is utilized by other microbes in the colon and the remaining is passed as flatus or excreted via breath (Christl, Murgatroyd, Cummings, & Gibson, 1992; Hylemon et al., 2018). Hydrogenotrophic (H_2–utilizing) microbes responsible for

converting hydrogen into other metabolites include 1) methanogens, which use H_2 as the electron donor to reduce CO_2 and produce CH_4; 2) sulfate reducing bacteria, which reduce sulfate to form H_2S; and 3) reductive acetogens that utilize the acetyl–CoA pathway to synthesize acetate from CO_2 and H_2 (Bernalier, Rochet, Leclerc, Doré, & Pochart, 1996; Drake, Gößner, & Daniel, 2008; Macfarlane & Gibson, 1997; Pimentel et al., 2013) (Table 1.2). These hydrogenotrophic bacteria prevent H_2 buildup in the colon that would thermodynamically inhibit fermentation and reduce the energy–extracting capacity of primary fermenters (Carbonero et al., 2012; Krajmalnik-Brown, Ilhan, Kang, K., & DiBaise, 2012).

Table 1.2. Gas-utilizing gut microbes

Classification	Genus and/or species	Gas utilized	By-product	Reference
Sulfate-reducing bacteria	*Desulfovibrio piger*	H_2	H_2S	(Rey et al., 2013)
	Desulfovibrio fairfieldensis	H_2	H_2S	(Ivan Kushkevych et al., 2019)
	Desulfovibrio desulfuricans	H_2	H_2S	(Ivan Kushkevych, 2016)
	Desulfobulbus	H_2	H_2S	(Dordević et al., 2021)
	Desulfomicrobium	H_2	H_2S	(Ivan Kushkevych, 2014)
	Desulfomonas	H_2	H_2S	(Ivan Kushkevych, 2014)
	Desulfovibrio piger	H_2	H_2S	(Rey et al., 2013)
Methanogens	*Methanobrevibacter smithii*	H_2 & CO_2	CH_4	(Weaver et al., 1986)

	Methanosphaera stadtmanae	H_2 & CO_2	CH_4	(Fricke et al., 2006)
	Methannobrevibacter oralis	H_2 & CO_2	CH_4	(Scanlan et al., 2008)
Reductive acetogens	*Ruminococcus*	H_2 & CO_2	Acetate	(Bernalier, Rochet, et al., 1996; Macfarlane & Gibson, 1997)
	Clostridium	H_2 & CO_2	Acetate	(Bernalier, Rochet, et al., 1996; Macfarlane & Gibson, 1997)
	Peptostreptococcus	H_2 & CO_2	Acetate	(Macfarlane & Gibson, 1997)
	Streptococcus	H_2 & CO_2	Acetate	(Bernalier, Rochet, et al., 1996)
	Blautia hydrogenotrophica	H_2 & CO_2	Acetate	(Bernalier, Willems, Leclerc, Rochet, & Collins, 1996; Liu, Finegold, Song, & Lawson, 2008)

1.2.2 Carbon dioxide

CO_2 is one of the major gases produced by colonic bacteria during the bacterial fermentation of carbohydrates in the colon (Montalto, Di Stefano, Gasbarrini, & Corazza, 2009). During fermentation, CO_2 can be produced from conversion of pyruvate to acetyl–CoA or cleavage of pyruvate to formate, which is then metabolized to H_2 and CO_2 by formate hydrogenlyase (Macfarlane & Gibson, 1997) (Fig. 1.1). Colonic bacteria, predominantly in the phyla Firmicutes and Bacteroidetes, are responsible for dietary carbohydrate fermentation and CO_2 production as a by–product of fermentation (Table 1.1).

Most CO_2 is passively absorbed by the colonic mucosa and enters into circulation through enterocytes and is then excreted via exhaled breath (Christl et al., 1992). Alternatively, unabsorbed CO_2 can be excreted in flatus or metabolized by resident microorganisms of the gut microbiome. For example, CO_2 can be reduced by hydrogenotrophic (H_2–utilizing) microbes such as methanogens in the presence of H_2 to produce CH_4, and by reductive acetogens that utilize the acetyl–CoA pathway to synthesize acetate from CO_2 and H_2 (Fig. 1.1 ; Table 1.2) (Drake et al., 2008; Fricke et al., 2006).

1.2.3 Hydrogen sulfide

SRB are anaerobic H_2 utilizers that are part of the human gut microbiota. SRB use H_2 as an electron donor to reduce sulfate and generate H_2S (Table 1.2). Sulfate may be derived in the gut from several foods, but is predominantly from sulfur–containing amino acids in proteins (Magee, Richardson, Hughes, & Cummings, 2000).

SRB numbers in the colon are approximately 10^3 to 10^{11} CFU/ gram of human stool (G. R. Gibson, Macfarlane, & Macfarlane, 1993). SRB are primarily the members of the *Desulfovibrio* genus of γ–proteobacteria (Pimentel et al., 2013). Particularly, *Desulfovibrio piger* is the most frequent SRB present in the colon (Rey et al., 2013). Other genera of colonic SRB include *Desulfotomaculum, Desulfobulbus*, *Desulfomicrobium, Desulfobacter,* and *Desulfomonas* (Barton & Hamilton, 2007; Kushkevych, 2016; Kushkevych & Moroz, 2012; Kushkevych, 2014).

The abundance of SRB and the level of H_2S accumulation in the human gut can have a health impact on the individual. High concentration of H_2S has toxic effects on human tissues (Ivan Kushkevych et al., 2019; Schicho et al., 2006). H_2S can impair metabolic action such as butyrate oxidation and protein synthesis. Furthermore, high levels of H_2S can cause DNA damage of epithelial cells (Attene-Ramos, Wagner, Gaskins, & Plewa, 2007). Consequently, several studies have suggested that sulfide is associated with intestinal disorders such as inflammatory bowel disease (IBD) (Gibson, Cummings, & Macfarlane, 1991).

1.2.4 Methane

Methanogens, group of microorganisms within the kingdom Euryarchaeota of the domain Archaea can utilize H_2 as electron donors to reduce CO_2 to CH_4 (Fig. 1.1) (Scanlan et al., 2008). This process of conversion of CO_2 and H_2 to CH_4 has the effect of reducing total gas volume by a factor of 5 ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$: 5 mol gas → 1 mol gas) (Bauchop & Mountfort, 1981; Blaut, 1994). Therefore, methanogenic metabolism can decrease gas volume and probably discomfort (Pimentel et al., 2013). However, methane production is considered a potential biomarker to diagnose symptoms

and disease in gastrointestinal disorders. High levels of CH_4 have been linked to decreased intestinal motility and are associated with constipation, especially in patients with irritable bowel syndrome with constipation (Chatterjee, Park, Low, Kong, & Pimentel, 2007; Kunkel et al., 2011; Pauff & Miller, 2012).

Substantial interindividual differences exist in colonic methanogenesis. The abundance of methanogens in human fecal samples varies from undetectable to 10^9 CFU per g of stool and a threshold population of 10^7 is required to result in detectable levels of CH_4 in the breath (Pochart, Doré, Lémann, & Rambaud, 1992). Studies indicate *Methanobrevibacter smithii* is the most abundant Archaeal species present in the gastrointestinal tract (GIT) (Eckburg et al., 2005; Weaver et al., 1986). Other methanogenic species in the GIT responsible for methane production are *Methanosphaera stadtmanae* and *Methanobrevibacter oralis* (Fricke et al., 2006; Triantafyllou, Chang, & Pimentel, 2014).

1.3 Food and intestinal gas

Although there are many causes of intestinal gas, food is one of the main causes influencing gas symptoms (Hasler, 2006; Tomlin et al., 1991). A large portion of people associate intake of certain foods with the development of intestinal gas and other gastrointestinal (GI) symptoms (Mego et al., 2015). This is especially true for foods rich in non–digestible, fermentable carbohydrates, proteins, and fats (Gibson, Varney, Malakar, & Muir, 2015). Recent studies have emphasized the relationship between gastrointestinal disorders and food intake, and many people, particularly those with gastrointestinal disorders want to know specific foods that can contribute to GI symptoms. Several studies have reported food groups and specific food items that cause

intestinal gas and other GI symptoms in patients with gastrointestinal disorders and healthy people. The most commonly reported flatulogenic food groups include pulses, vegetables, fruits, whole grains, and dairy products (MacDermott, 2007; Manichanh et al., 2014b).

1.3.1 Pulses

Pulse foods including dry beans, lentils, peas, and others are an important staple food for many people across the world because of their nutritional, economic, and health benefits. Pulses are generally low in fat and an inexpensive rich source of proteins, vitamins, fibers, and minerals (Perera, Russo, Takata, & Bobe, 2020). However, despite all these benefits, many consumers avoid eating pulses, especially a wide variety of beans, because of the fear of excessive flatulence and stomach discomfort (Descrochers & Brauer, 2001; Fleming, O'Donnell, & Perman, 1985). It has been reported that increased flatulence is an expected outcome among some people after the inclusion of pulses in their diet, especially if fiber intake is already low (Perera et al., 2020; Tomlin et al., 1991). However, it has been reported that flatulence does decrease with more frequent consumption of pulses (Livesey, 2001). In a randomized controlled trial, 50% of healthy subjects reported increased flatulence in the first week of consuming pinto or baked beans, but the reported percentage dropped to 38% after the second week (Winham & Hutchins, 2011). Thereafter the percentage of people reporting flatulence symptoms consistently declined to 15–23% for weeks 6–12. Similarly, in another randomized controlled trial, the impact of 28 consecutive days of consumption of pulses (chickpeas, lentils, and green peas) was assessed (Veenstra et al., 2010). A significant increase in flatulence in the early phase of the intervention was observed with the consumption of

each pulse, but the level of flatulence significantly declined in the late phase. Based on these studies, the frequency of passing gas depends on the how frequently an individual consumes pulses, and the response can vary significantly from one individual to another.

Most pulses contain relatively high amounts of both dietary fibers and resistant starches. Furthermore, they are particularly rich in soluble, fermentable oligosaccharides that belong to the raffinose family of oligosaccharides (RFOs) (Table 1.3) (Elango et al., 2022). RFOs are non–reducing carbohydrates consisting of one to several 1→6–linked α–galactopyranosyl units linked to C–6 of the glucose moiety of sucrose (Andersen, Bjergegaard, Møller, Sørensen, & Sørensen, 2005). Raffinose, stachyose, and verbascose are the common RFOs (Van den Ende, 2013). Raffinose and stachyose exist ubiquitously in plants, whereas verbascose is found in the vacuoles of only certain plants (Elsayed, Rafudeen, & Golldack, 2014). These RFOs cannot be digested or absorbed in the small intestine due to the lack of α–galactosidase enzymes in humans to degrade RFOs (Kalantar-Zadeh et al., 2019). Therefore, once RFOs reach the colon, they undergo fermentation by colonic bacteria that produce gases as metabolic byproducts (Price, Lewis, & Fenwick, 1988). Consequently, consumption of legumes can cause increased flatulence in some people (Lacy, Gabbard, & Crowell, 2011; Naczk, Amarowicz, & Shahidi, 1997). Many studies have found that the removal of RFOs from plants through processing methods such as extrusion, soaking, autoclave, enzyme use, and boiling can reduce RFOs; and presumably reduce gas production.

Table 1.3. Variability in raffinose family oligosaccharides (mg/g) present in dry seeds of various plants
(Elango et al., 2022; Price et al., 1988).

Crop	Raffinose	Stachyose	Verbascose
Dry Peas	4.1–10.3	10.7–26.7	0.0–26.7
Soybean	11.1.–18	31–48	–
Chickpea	8.1–9	15–19	–
Lentil	28.6–37	24.6–28.8	3.9–7.2
Green peas	30.1	35.4	15
Cowpea	12	34	9
Mung bean	4.1–5	17–20	–
Peanut	3	9	–
Red kidney bean	3.1	31.6	–
Green bean	2.5	34.3	–
Faba bean	2.3	10.7	11.4
Black eyed peas	4	4	–
Lima bean	6.9	30.3	–

1.3.2 Vegetables and fruits

Vegetables that are high in fructans have been reported to be associated with increased flatulence in humans after consumption (Bruhwyler, Carreer, Demanet, & Jacobs, 2009; Grabitske & Slavin, 2009). The fructans in vegetables and fruits are classified as inulin-type, which consist of one to several 2→1–linked β- fructofuranosyl units linked to the fructose moiety of sucrose. The simplest fructan of this type is 1-kestose, which is β-fructofuranosyl-(2→1)-β-fructofuranosyl-(2→1)-α-glucopyranoside. Degrees of polymerization of inulin-type fructans usually ranges from 3 to about 60 (Cooper et al., 1996).

Inulin and FOS are abundant in vegetables such as artichokes, asparagus, chicory root, garlic, onions, dandelion greens and leeks (Table 1.4). Fructans cannot be digested

in the small bowel and result in excessive intestinal gas after bacterial fermentation in the colon. Two recent studies examined the effect of consuming inulin–containing foods on gastrointestinal symptoms. The findings from both studies showed that the most frequent gastrointestinal symptoms reported by healthy volunteers after consuming inulin–rich diets was increased flatulence (Holscher et al., 2014; J. Slavin & Feirtag, 2011). In another study, FOS was reported to contribute to increasing the production of intestinal gas during 5 weeks of consumption of a FOS–rich diet (Alles et al., 1996).

Table 1.4. Inulin and fructooligosaccharide contents of selected vegetables (Alles et al., 1996; Holscher et al., 2014; Loo, Coussement, De Leenheer, Hoebreg, & Smits, 1995; Moshfegh, Friday, Goldman, & Chug Ahuja, 1999; Sabater-Molina, Larqué, Torrella, & Zamora, 2009; J. Slavin & Feirtag, 2011).

	Inulin (g/100g)	Fructooligosaccharides (g/100g)
Jerusalem artichoke	16.0–20	12.0–15.0
Globe artichoke	1.2–6.8	0.2–0.7
Chicory roots	35.7–47.6	19.6–26.2
Dandelion greens		
Raw	12.0–15.0	9.6–12.0
Cooked	8.1–10.1	6.5–8.1
Garlic	9.0–16	3.6–6.4
Onion		
Raw	1.1–7.5	1.1–7.5
Cooked	0.8–5.3	0.8–5.3
Leeks	3.0–10.0	2.4–8.0
Asparagurus		
Raw	2.0–3.0	2.0–3.0
Cooked	1.4–2.0	1.4–2.0

Sugar alcohols, also known as polyols, are found naturally in some fruits and vegetables (Table 1.5) and have also been implicated in intestinal gas due to their poor absorption in the small intestine (Grembecka, 2015; Langkilde, Andersson, Schweizer, & Wursch, 1994; Lenhart & Chey, 2017). For example, one study assessed the association between sorbitol malabsorption and gastrointestinal symptoms when 7 healthy

participants consumed different doses of sorbitol (Hyams, 1983). In a majority of subjects (4 of 7), ingestion of as little as 5 g of sorbitol was associated with significant increase in gas, while most subjects experienced severe symptoms (gas, bloating, cramps, and diarrhea) after consuming 20 g of sorbitol. Similarly in another study with 10 healthy volunteers, ingestion of hydro solution containing high dose of mannitol led to the highest rate of side effects, including flatulence, diarrhea, and abdominal pain compared to the ingestion of low dose of mannitol (Ajaj et al., 2004). Both these studies conclude that the malabsorption of sugar alcohols causes flatulence among participants, but the severity of the symptom depends on the dose of polyols.

Polyols are also commonly used as sugar–free sweeteners in chewing gums and beverages (Yao et al., 2014). Examples of polyols approved by the US FDA are mannitol, sorbitol, xylitol, isomalt, lactitol, maltitol, erythritol, and hydrogenated starch.

Table 1.5. Sorbitol and mannitol concentration of selected vegetables and fruits.

Category	Sorbitol (g/100g)	Mannitol (g/100g)
Vegetables		
Brussel sprouts	0.2	0
Broccoli	0.3	0
Cabbage	0.2	0
Cauliflower	0	2.6
Celery	0	1.5
Mushrooms	0.1	2.6
Sweet potatoes	0	0.3
Fruits		
Plum	2.4	0
Apple	1.2	0
Apricot	1.2	0
Blackberries	4.1	0
Cherries	0.7	0
Nectarine	1	0
Peach	0.9	0.5
Pear	2.3	0

1.3.3 Whole grains

Grains such as wheat, corn, barley, rye, and oats are staple foods that most of the world's population rely on as the main proportion of the diet. However, nutritionists and governmental agencies recommend consuming whole rather than refined grains. The 2020–2025 Dietary Guidelines for Americans recommend eating at least 3 ounces of whole grains/day due to the associated health benefits of whole grain consumption. Whole grains contain some soluble fibers and resistant starch that can be fermented, but mostly contain poorly fermentable carbohydrates such as cellulose and cross–linked arabinoxylan (Nirmala Prasadi & Joye, 2020). However, whole grains also contribute a significant quantity of fructans to diets (Table 1.6). The fructans in grains are different from those found in fruits and vegetables. They are classified as levan–type (Gallagher, Cairnsz, Turne, & Gallagher, 2007; van den Ende et al., 2011; Yoshida & Tamura, 2011), which contain β-fructofuranosyl units linked by (2→6) bonds to sucrose, as in 6-kestose [β-fructofuranosyl-(2→6)-β-fructofuranosyl-(2→1)-α-glucopyranoside] (Roberfroid & Delzenne, 1998; Rossi et al., 2005). Levans from grains have been reported to reach about 90 degrees of polymerization (Roberfroid & Delzenne, 1998). Although the concentrations of fructans in grains are not high, wheat is the major source of naturally occurring fructans in the diets of Americans due to the frequent consumption of wheat (Moshfegh et al., 1999).

Table 1.6. Inulin and fructooligosaccharide contents of selected grains (Alles et al., 1996; Holscher et al., 2014; Loo et al., 1995; Moshfegh et al., 1999; Sabater-Molina et al., 2009; J. Slavin & Feirtag, 2011).

Grain	Inulin (g/100g)	Fructooligosaccharides (g/100g)
Wheat		
Bran–raw	1.0–4.0	1.0–4.0
Flour–baked	1.0–3.8	1.0–3.8
Flour–boiled	0.2–0.6	0.2–0.6
Barley		
Raw	0.5–1	0.5–1.0
Cooked	0.1–0.2	0.1–0.2
Rye		
Baked	0.5–0.9	0.5–0.9

A substantial amount of research has linked whole grain consumption to increased flatulence. In an uncontrolled study, 55% of IBS patients reported that bran made their gastrointestinal symptoms worse including gaseous complaints (Francis & Whorwell, 1994). Controlled trials of wheat bran have also reported increased flatulence and abdominal discomfort in participants compared to placebo (Cann, Read, & Holdsworth, 1984). Additionally, Vuholm et al. investigated whether whole–grain wheat and whole–grain rye affect gastrointestinal (GI) symptoms and found that intake of these whole grains was significantly associated with increased flatulence in healthy volunteers (Vuholm et al., 2017).

Although, fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) such as fructans found in wheat and other grains are commonly known to cause severe gastrointestinal symptoms in IBS patients, it has been also reported that many people with non-Celiac gluten sensitivity may in fact be sensitive to FODMAPs in wheat rather than gluten (Molina-Infante, Santolaria, Montoro, Esteve, & Fernández-Bañares, 2014). Consequently, several studies have evaluated different methods to reduce fructans in whole grain foods, which can presumably decrease

intestinal gas and other gastrointestinal symptoms. One study evaluated the ability of different sourdough strains of yeast to degrade fructans in wheat flour during 96 hours of fermentation. After the fermentation, *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* isolated from Australian sourdough demonstrated the ability to significantly reduce the fructan content in the wheat flour compared to traditional baker's *S. cerevisiae* isolates ((Fraberger, Call, Domig, & D'Amico, 2018). In another study, Pejcz et al, also investigated the potential of *Lactobacillus plantarum* with extended fermentation time with *Saccharomyces cerevisiae* to degrade fructans in rye bread during rye dough fermentation. After 3 hours of fermentation, the authors noticed that the content of fructans in bread was significantly decreased in rye bread fermented with *L. plantarum* and yeast than in bread fermented with baker's yeast alone (Pejcz et al., 2020). Therefore, based on these studies, the use of *Lactobacillus* strains and sourdough yeast can be an effective method to reduce the content of fructans in whole grain bread.

1.3.4 Dairy products

Milk and other dairy products are among the food items that aggravate GI symptoms among the considerable proportion of the adult population of the world that is deficient in lactase, an enzyme responsible for the hydrolysis of lactose, the primary carbohydrate in dairy foods (Campbell, Waud, & Matthews, 2009). In most mammals, lactase activity is high in children but decreases rapidly with age; however, in humans, 30% of the world population are lactase–persistent as adults, but the prevalence of lactase persistence varies widely in frequency across the human population (Bayless, Brown, & Paige, 2017; Montgomery, Krasinski, Hirschhorn, & Grand, 2007). Lactose is a unique carbohydrate present in mammalian milk, 7.2g/100ml in human milk and 4.7 g/100ml in

cow's milk (Solomons, 2002). When lactose is not hydrolyzed into its simple sugars (glucose and galactose) in the small intestine, it enters the large intestine where it serves as a fermentable substrate for colonic bacteria (Campbell et al., 2009). It is reported that patients suffering from lactase deficiency experience increased flatulence as a result of the fermentation of lactose in the colon (Le Nevé et al., 2019).

1.4 Conclusion

The gaseous by–products of microbial fermentation can have both direct (e.g., IBD, constipation, and DNA damage) and indirect (abdominal pain and bloating) effects on the host. It is clear that the presence of different strains of bacteria in the colon and various types of food rich in non–digestible carbohydrates play a huge role in aggravating gastrointestinal symptoms, especially increased flatulence, in patients with gastrointestinal disorders and healthy people. Therefore, understanding gut microbiome composition, gaseous products released by these microbiomes, and foods that influence gas may enhance the knowledge to mitigate the symptoms linked to intestinal gas.

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CHAPTER 2 THE ROLE OF *MEGASPHAERA ELSDENII*, A COMMENSAL MEMBER OF THE GUT MICROBIOTA, IN GAS PRODUCTION DURING *IN VITRO* FERMENTATION

ABSTRACT

Gas production and bloating remain significant barriers to increasing intake of dietary fiber containing foods for many consumers. Previously, we found that an amplicon sequence variant (ASV) from *Megasphaera elsdenii* (*M. elsdenii* 4415), was correlated with high gas production during fermentation of pulses. Therefore, the objective of this study was to determine the role of *M. elsdenii* 4415 in gas production by the microbiome during fermentation of kidney beans and sweet potatoes—two foods commonly associated with gas production and bloating. Sequencing data from 29 fecal samples were examined for the presence of *M. elsdenii* 4415. Among these stool samples, three were positive for *M. elsdenii* 4415 and comprised a *M. elsdenii* 4415-positive group (Me+). Seven microbiomes that were negative for *M. elsdenii* 4415 (Me–) and came from donors with similar intakes of kidney beans and sweet potatoes to the (Me+) microbiomes were selected as a control group. Kidney beans and sweet potatoes were subjected to *in vitro* digestion to remove digestible components and then used the residue as a substrate for *in vitro* fermentation using the selected microbiomes. The primary outcome was gas production during fermentation. The Me+ microbiomes produced significantly more gas than the Me− microbiomes after 24 h of fermentation of sweet potatoes (10.4±0.2 mL versus 7.4±0.65 mL, $p<0.001$), and by 48 h of fermentation the Me+ microbiomes produced more gas than the Me− microbiomes regardless of substrate

(kidney beans: 14.0±2.3 mL versus 10.1±0.8 mL, $p<0.001$; sweet potatoes: 23.0±0.9 mL versus 7.76±0.84 mL, $p<0.001$). In addition, *M. elsdenii* 4415 relative abundances increased dramatically among Me+ microbiomes on both beans and potatoes—up to 60% in some microbiomes after 48 h of fermentation. There were four other ASVs that were significantly associated with gas production; however, *M. elsdenii* 4415 had the strongest association with gas after 48 h of fermentation. Me+ microbiomes resulted in increased butyrate production compared to Me− microbiomes, while Me− microbiomes resulted in higher acetate and propionate production after fermentation. This study suggests *M. elsdenii* is a commensal member of the microbiome that may be responsible for high gas production during fermentation of flatulogenic foods. Furthermore, in this study gas production was primarily generated through cross-feeding on acetate (and possibly lactate) and not produced directly from metabolism of non-digestible carbohydrate.

2.1 Introduction

Often flatulence is a regular, everyday occurrence that can be awkward in social circumstances where it is perceived as embarrassing. However, even this regular flatulence can be a sufficient deterrent for some people to consume foods that are associated with flatulence (Szczebyło, Rejman, Halicka, & Laskowski, 2020). Furthermore, sometimes intestinal gas becomes severe and can cause distress, discomfort, and chronic conditions such as irritable bowel syndrome (MacDermott, 2007).

The volume of intestinal gas produced among individuals differs from one another, ranging from as little as 0.2 L to as much as 1.5 L per day (Mego, Accarino, Malagelada, Guarner, & Azpiroz, 2015; Serra, Azpiroz, & Malagelada, 1998; Suarez, Springfield, & Levitt, 1998). The main factors influencing the variation in intestinal gas volume produced among individuals are diet and gut microbiota composition (Manichanh et al., 2014b). Many people associate the intake of certain foods with high gas production (Mego et al., 2015). Typically these foods are high in dietary fibers (Bolin & Stanton, 1998). The dietary fibers, especially the short-chain, oligomeric dietary fibers found in these foods, escape digestion and absorption in the small intestine and are fermented by gut bacteria in the colon to yield the flatus gases methane, hydrogen, and carbon dioxide (Mego et al., 2015). Examples of such foods include kidney beans, sweet potatoes, lentils, chickpeas, soybeans, and peas (Den, Biermann, & Marlett, 1986; Veenstra et al., 2010; D. M. Winham & Hutchins, 2011). In particular, the consumption of kidney beans has decreased in recent years, largely because of the belief that kidney beans may cause increased flatulence (Lim et al., 2014).

Many bacteria in the human gut are responsible for flatulogenic gases produced during the fermentation of non-digestible substrates. These bacteria mostly belong to the Bacteroidota (formerly Bacteroidetes) and Firmicutes phyla, which together usually make up 90% or more of the total bacterial population in the human gut (Arumugam et al., 2013; Rinninella et al., 2019). However, there may be "keystone" members of the microbiota that, if present, are responsible for unusually high gas production relative to microbiomes lacking these keystone members (Banerjee, Schlaeppi, & van der Heijden, 2018).

In support of this speculation, in a previous study gas production was quantified during fermentation of processed pulses using fecal microbiotas from healthy donors (Fig. 2.1A) (Rose et al., 2021). There were three amplicon sequence variants (ASV) belonging to *Clostridium sensu stricto* cluster 1, *Dialister*, and *Megasphaera* that were significantly correlated with gas production during the fermentations. The *Megasphaera* ASV, which was classified as *M. elsdenii*, a member of the Firmicutes phylum, was particularly interesting because in one microbiome that produced about 2-fold more gas than all of the other microbiomes it was present at unusually high abundance (20% in the fecal inoculum increasing to 50–75% during 24 h of fermentation) (Fig. 2.1B). The relative abundance of the other ASVs belonging to *Clostridium sensu stricto* cluster 1 and *Dialister* were more common among all microbiomes and present at much lower abundances throughout fermentation (Fig. 2.1C, D). Thus, it was speculated that *M. elsdenii* may be a "keystone" member of the microbiota responsible for elevated gas production during fermentation.

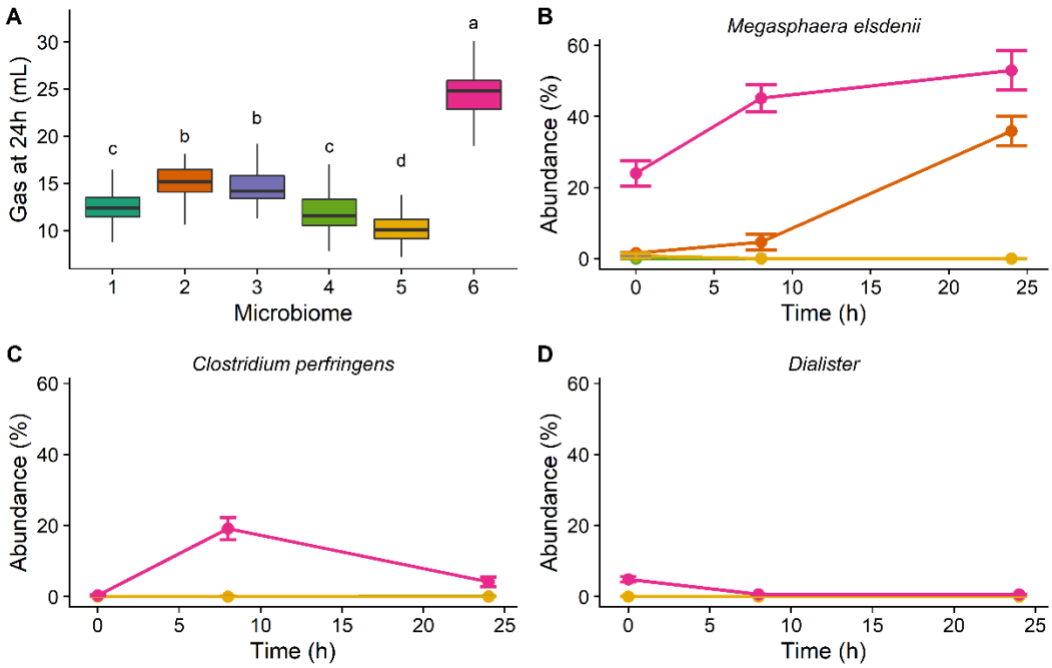

Figure 2.1. Gas production by 6 microbiomes after 24 h of fermentation (A); relative abundances of the 3 ASVs that were positively correlated with gas production during 24 h of fermentation (B-D) (Rose et al., 2021).

M. elsdenii is a gram-negative coccoid-shaped obligate anaerobe and has been used as a probiotic in ruminants because of its ability to metabolize lactate to short chain fatty acids, mainly butyrate, in the prevention of rumen acidosis (Aikman, Henning, Humphries, & Horn, 2011; Chen et al., 2019; Shetty, Marathe, Lanjekar, Ranade, & Shouche, 2013). However, some studies examining the efficacy of *M. elsdenii* as a probiotic feed supplement have also measured gas production and found that it is increased in animals supplemented with *M. elsdenii* (Sedighi & Alipour, 2019). *In vitro* fermentation using horse gut microbiome has shown that addition of *M. elsdenii* causes a significant increase in gas production during fermentation of inulin and corn starch (Douthit et al., 2019b).

Although lactate accumulation and utilization are not well known in the human gut, lactate has been reported to accumulate in the fecal matters of individuals with short bowel syndrome and ulcerative colitis, at concentrations up to 100 mM, while individuals with no apparent disease have less than 5mM fecal lactate (Hove, Nordgaard-Andersen, & Mortensen, 1994; Kaneko et al., 1997). High lactate accumulation may induce neurotoxicity and cardiac arrhythmia. *M. elsdenii* may maintain the balance of human gut lactate by metabolizing lactate to butyrate and other SCFAs, but also increase gas production (Duncan, Louis, & Flint, 2004; Jiang, Su, & Zhu, 2016).

M. elsdenii is only sparsely present among human gut microbiotas (Duncan et al., 2004). *M. esldenii* was reported to be in 10% of healthy human fecal samples (Sugihara, Sutter, Attebery, Bricknell, & Finegold, 1974; Werner, 1973). While *M. elsdenii* may offer health benefits to hosts that harbor it through its conversion of lactate to butyrate, a beneficial microbial metabolite, it may also cause increased intestinal gas production and lead to intestinal discomfort. Therefore, the purpose of this study was to investigate the role of *M. elsdenii* in gas production during *in vitro* fermentation of two flatulogenic foods, red kidney beans and sweet potatoes.

2.2 Materials and Methods

2.2.1 Fecal sample selection

As explained in the Introduction, our previous study identified one ASV that was particularly interesting for its relationship to gas production (Rose et al. 2021). The Quantitative Insights into Microbial Ecology (QIIME) ID assigned to this ASV was 44158349d8858abc6c04aada0c131da5 and it was classified as *M. elsdenii*; therefore, it was named '*M. elsdenii* 4415'.

For fecal sample selection, sequencing data from 29 fecal samples collected from two previous studies were examined for the presence of *M. elsdenii* 4415. There were four microbiomes that were positive for *M. elsdenii* 4415 and were therefore selected as the *M. elsdenii* positive group (Me+). Six microbiomes that were negative for *M. elsdenii* 4415 (Me–) were selected as a control group. Because the purpose of this study was to examine the effects of *M. elsdenii* 4415 on gas production during fermentation of sweet potatoes and kidney beans, the six microbiomes that made up the Me– group were selected based on having a diverse diet, that was also not significantly different for sweet potatoes and kidney beans compared to Me+ group. Intake of sweet potatoes and kidney beans was estimated from responses to the Diet History Questionnaire III (National Cancer Institute-Division of Cancer Control & Population Sciences., 2020), which all stool donors completed at the time of fecal collection.

The fecal samples were prepared by mixing each fecal sample with anaerobic sterile phosphate-buffered saline, pH 7.0, containing 10% glycerol as a cryoprotectant at 1:9 w/v inside a sterile filter bag (Filtra-Bag, Thomas Scientific, New Jersey) within 2 h of defecation. A stomacher was used to homogenize each fecal slurry for 4 min, and then the mixture was transferred to an anaerobic chamber (containing 5% H_2, 5% CO_2, and 90% N_2, Bactron X, Sheldon Manufacturing, Cornelius, OR, USA) and aliquoted in 15 mL polypropylene centrifuge tubes. Fecal slurries were then stored at −80 °C until further use. The procedures involving human subjects were approved by the Institutional Review Board of the University of Nebraska before initiating the study (20210621206EP, 20200219980FB).

As described, fecal samples were categorized into Me+ (n = 4) and Me− (n = 6) based on the presence or absence of *M. elsdenii* 4415 in fecal samples previously collected (Fig. 2.2A). However, when sequencing data was analyzed for this study, one microbiome that was originally classified as Me+ (RS514) had no reads for *M. elsdenii* 4415. Therefore, RS514 was re-classified as Me− for a final distribution of n = 3 for Me+ and n = 7 for Me−(Fig. 2.2A). With the new groupings, Me− microbiomes still had a diverse diet covering all quadrants of the PC biplot (Fig. 2.2B), but also there were still no significant differences between Me groups for potato and legume intake of fecal donors (Fig. 2.2C).

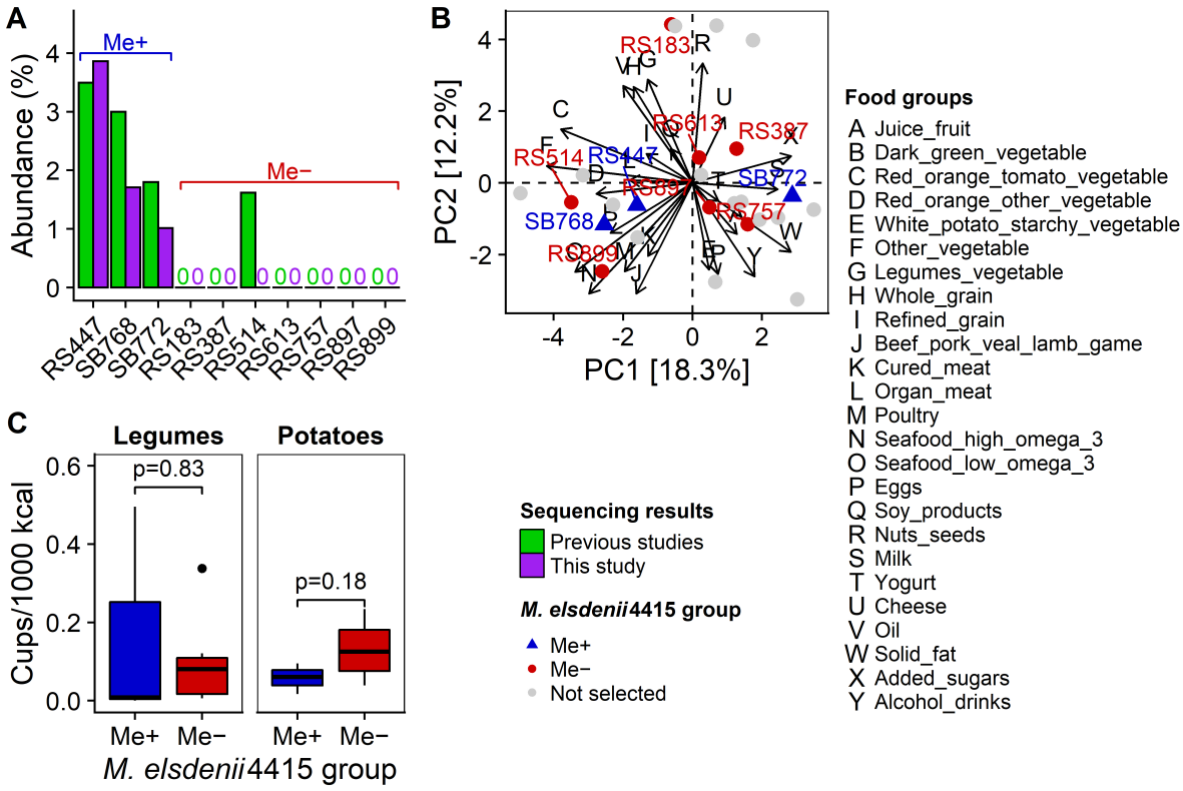

Figure 2.2. *M. elsdenii* 4415 fecal abundances of the ten selected fecal samples from sequencing results of both the previous study and this study, only 3 fecal samples were positive for *M. elsdenii* for this study (A); Principal components (PC) biplot of dietary nutrient intakes (DHQ3) of ten selected microbiomes including 3 microbiomes that were positive for *M. elsdenii* (Me+) and the 7 microbiomes that were negative for *M. elsdenii* (Me−) that were selected for in vitro fermentation of kidney beans and sweet potatoes (B). Legume and potato intake of the selected Me+ and Me− respondents (C).

2.2.2 Substrate preparation

White flesh sweet potatoes (*Ipomoea batatas*) and red kidney beans (*Phaseolus vulgaris*) were purchased from a local market. Sweet potatoes were peeled, cut into cubic pieces (2.0 cm X 2.5 cm), covered with distilled water to a depth of 2.5 cm. The mixture was then brought to boiling and then simmered (85-95 °C) for 25 min. After sweet potatoes were fully cooked, they were drained and allowed to cool to room temperature. The sample was then blended in a food processor (2.5Qt Pro Commercial, Waring, McConnellsburg, PA) for 1 min. The homogenized sweet potatoes were transferred to zip-top storage bags, frozen, and then freeze-dried (FreeZone Tray Dryer, Labconco, Kansas City, MO, USA) before storage at −80°C for further use.

Red kidney beans were soaked in distilled water at 1:5 (w/v) for 16 h at room temperature. The distilled water was then discarded, and the soaked kidney beans were transferred to a pot filled with fresh distilled water (1:10, w/v). The mixture was then brought to boiling and then simmered (85-95 °C) for 1 h. After cooking, kidney beans were drained and blended in a food processor (Waring) for 1 min. The homogenized beans were transferred to zip-top storage bags, frozen, and then freeze-dried before storage at −80°C for further use.

2.2.3 *In vitro* digestion

Freeze-dried sweet potatoes and kidney beans were subjected to *in-vitro* digestion as described (Bengtsson, Alminger, & Svanberg, 2009), with some modifications. Briefly, 3 g of freeze-dried sample was weighed into a 50 mL centrifuge tube. Then, 10 mL of simulated salivary fluid [50 mM NaCl, 10 mM KH_2PO_4, 2 mM $CaCl_2 \cdot 2H_2O$, 40 mM $NaHCO_3$ containing 1 mg/mL α-amylase (1000 U/mg, Sigma-Aldrich, St. Louis,

MO, USA)] were added, and the pH was adjusted to 6.7 with 1M $NHCO_3$. The slurry was incubated for 15 min at 37 °C in a water bath with reciprocal shaking at 100 rpm. Next, the pH was reduced to 2 with 1 M HCl before adding 5 mL of simulated gastric fluid [50 mM NaCl, 14 mM KCl, 3.5 mM KH_2PO_4, 10 mM $CaCl_2 \cdot 2H_2O$, 3.6 mM $MgCl_2 \cdot 6H_2O$, containing 21 g pepsin /L (914 units/g solids, P-7000; Sigma-Aldrich, St. Louis, MO, USA)] and the mixture was incubated for 30 min at 37 °C with shaking at 100 rpm. To simulate the intestinal phase, the pH was raised to 6.9 with 1M $NHCO_3$, then 3 mL of pancreatin/bile solution [4.5 g/L pancreatin (P-7545; Sigma-Aldrich) and 28 g/L bile salts (Oxoid, Cheshire, England) in 100 mM $NaHCO_3$] were added and the mixture was incubated for 2 h at 37°C with orbital shaking at 100 rpm. Following digestion, the slurry was transferred into dialysis tubing (MWCO 100-500 Da; Spectra Por 131060) and dialyzed for 72 h in distilled water at 4°C that was changed every 3 hours during the day (4 times/day). The retentate was then freeze-dried and stored at −80 °C.

2.2.4 *In vitro* fermentation

In vitro fermentation was performed as previously described (Yang & Rose, 2014) with some modifications. Briefly, inside the anaerobic chamber, 40 mg of freeze-dried beans or sweet potatoes obtained after *in vitro* digestion and dialysis were suspended in 4 mL of sterile anaerobic fermentation media in a Hungate tube. The fermentation medium contained (per L): 2 g peptone (Fisher Scientific, Waltham, MA, USA), 2 g yeast extract (Fisher Scientific, Waltham, MA, USA), 0.5 g bile salt (Oxoid, Cheshire, England), 2 g $NaHCO_3$, 0.1 g NaCl, 0. 5g L-cysteine (Fisher Scientific, Waltham, MA, USA), 2 mL Tween 80 (Fisher Scientific, Waltham, MA, USA), 1 mL Vitamin K solution (10 µL/1 mL dissolved in ethanol; Alfa Aesar, Haverhill, MA, USA),

4 mL of resazurin solution (1 mg/4 mL dissolved in water; Alfa Aesar, Haverhill, MA, USA), 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 1 mL hemin solution (0.015 g hemin dissolved in 3 mL DMSO), and 0.04 g K_2HPO_4. Hungate tubes were then inoculated with 0.4 mL of fecal slurry, and immediately sealed with a rubber stopper and aluminum seal. Then, tubes were incubated in a water bath at 37°C with orbital shaking at 60 rpm. The gas volume produced during fermentation was measured after 24 h and 48 h by inserting a needle attached to a glass syringe through the septum and reading the gas volume from the graduations on the syringe. After gas measurements, the fermented samples were aliquoted in 1.5 mL centrifuge tubes and stored at −80 °C. All fermentations were performed in triplicate with separate tubes for 0 h, 24 h, and 48 h measurements.

2.2.5 Short chain fatty acids

The fermented samples were thawed and centrifuged at 9600*g* for 10 min. The supernatants was then collected and used for SCFA analysis as previously described (Hartzell, Maldonado-Gómez, Hutkins, & Rose, 2013). In short, 0.4 mL of supernatant was vortex mixed with 0.1 mL of 7 mM 2-ethylbutyric acid in 2 M potassium hydroxide, 0.2 mL of 9 M sulfuric acid, and ~ 0.1 g of sodium chloride in a 2 mL screw cap microcentrifuge tube. Diethyl ether (0.5 mL) was then added, and the mixture was inverted several times followed by centrifugation at 13600g for 1 min. The top layer was collected and injected into a gas chromatograph (Clarus 580; PerkinElmer, Waltham, MA, USA) equipped with a capillary column [Nukol; 30 m (l) × 0.25 mm (i.d.) × 0.25 μm (film thickness); Supelco, Bellefonte, PA] and a flame ionization detector. The quantification of SCFA was done by calculating response factors for each short fatty acid

(acetate, propionate, butyrate, iso-butyrate, and iso-valerate) relative to 2-ethylbutyric acid using injection of pure standards.

2.2.6 Microbiota Composition

Bacterial DNA was extracted from bacterial pellets obtained from SCFA analysis using the BioSprint 96 workstation (Qiagen, Germantown, MD), Biosprint 96 One-For-All Vet kit, stool lysis buffer ASL (Qiagen, Germantown, Maryland), and bead beating. The amplicon sequencing of the V4 region of the bacterial 16S rRNA gene was completed using the Illumina MiSeq platform and the MiSeq reagent kit v2 (2 × 250 bp) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Sequences were demultiplexed and barcodes were removed prior to sequence analysis with QIIME 2 (Bolyen et al., 2019). Sequence quality control, trimming, chimera removal, and denoising were performed with DADA2 (Callahan et al., 2016). Forward and reverse reads were truncated to 245 and 160 bp, respectively, to maintain sequence qualities above a phred score of 30. Using DADA2, sequences were dereplicated into 100% ASVs for exact sequence matching. Taxonomy was assigned using the SILVA database (Quast et al., 2013). Samples were normalized to the median sequence depth of 25037 reads/sample prior to diversity calculations. Then, for statistical analysis, low abundance spurious sequences were filtered by removing taxa with total number of reads of <0.25% in all samples prior to statistical analysis (Reitmeier et al., 2021). Normalization, diversity calculations, and filtering were performed using the phyloseq package in R (version 4.1.3) (McMurdie & Holmes, 2013).

2.2.7 Data analysis

All data were analyzed using R (version 4.1.3) and RStudio (2022.02.3 Build 492) with various packages as described. Gas production and short chain fatty acids were

analyzed using a two-way nested ANOVA by time (24 h and 48 h), where substrate (sweet potatoes or kidney beans) and microbiome nested within *M. elsdenii* group (Me+ or Me−) were the factors. Tukey's test was performed to determine significant differences between levels of the substrate X *M. elsdenii* group interaction at each time point. *M. elsdenii* 4415 relative abundance was analyzed using the Kruskal-Wallis test followed by Dunn's test to find significant differences among substrates and *M. elsdenii* groups at each time point. For microbiota composition data, Multivariate Association with Linear Models 2 (MaAsLin2) was used to identify ASVs that were associated with gas production during fermentation (Mallick et al., 2021). The MaAsLin2 model treated gas production as a fixed effect and microbiome, substrate, and time as random effects. Beta diversity analysis was performed using constrained analysis of principal coordinates (CAP) biplot based on the Bray-Curtis distance matrix. Then, PERMANOVA analysis was conducted to examine whether the composition of Me+ microbiomes was different from Me− microbiomes, using the "Adonis" command in vegan package in R with 99 permutations (Oksanen et al., 2022). The vector for gas production was added to the CAP biplot by correlating gas production with Eigenvalues from the CAP analysis and using these as the X and Y coordinates.

2.3 Results

2.3.1 Gas production by microbiomes

Since many consumers associate the intake of dietary fiber-containing foods with increased gas production, two flatulogenic foods, red kidney beans and sweet potatoes were subjected to *in vitro* fermentation using ten selected microbiomes to identify the role of gut bacteria, particularly *M. elsdenii* in gas production. The Me+ microbiomes

produced significantly more gas than the Me− microbiomes after 24 h of fermentation of sweet potatoes; however, by 48 h of fermentation the Me+ microbiomes produced more gas than the Me− microbiomes regardless of substrate (Fig. 2.3). Me+ microbiomes that were treated with digested sweet potatoes as substrate produced significantly more gas than those treated with kidney beans at both 24 h and 48 h of fermentation, while the opposite was true for the Me− microbiomes.

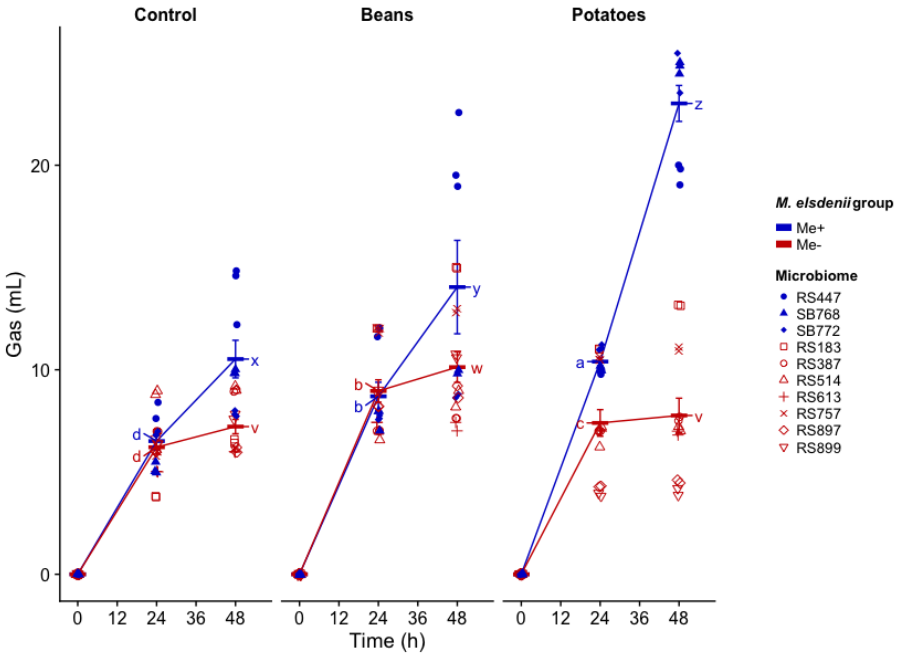

Figure 2.3. Gas production during 48 h of fermentation of kidney beans or sweet potatoes. Error bar shows standard error; different letters denote significant differences among substrates and *M. elsdenii* 4415 groups at the same time point (Tukey's HSD $p<0.05$).

During fermentation, *M. elsdenii* 4415 relative abundances increased dramatically among Me+ microbiomes on both beans and potatoes—up to 60% in some microbiomes (Fig. 2.4). Although Me− microbiomes had no detected *M. elsdenii* 4415 in the stool samples (0 h of fermentation), *M. elsdenii* 4415 was detected during fermentation in some microbiomes in this group, but at a lower relative abundance (<2%, except one

outlying replicate from RS387 at 24 h on sweet potato substrate with 28% abundance) compared to Me+ microbiomes (Fig. 2.4).

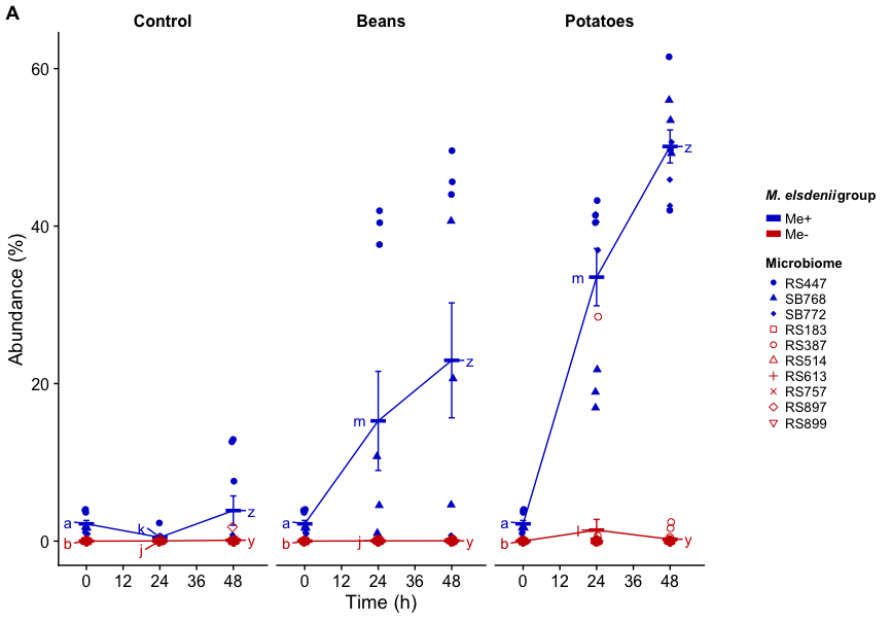

Figure 2.4. *M. elsdenii* (Me) relative abundance during 48 h of fermentation of kidney beans and sweet potatoes by microbiomes positive for *M. elsdenii* (Me+) and microbiomes negative for *M. elsdenii* (Me−). Different letters denote significant differences among substrates and *M. elsdenii* 4415 groups at the same time point (Dunn's test $p<0.05$).

A constrained analysis of principal coordinates biplot based on Bray-Curtis distance among the samples showed that the microbiome composition of Me+ microbiomes was significantly different from Me− microbiomes (Fig. 2.5). The Eigenvector for *M. elsdenii* 4415 pointed strongly toward the samples from the Me+ microbiomes, as expected. When gas production was overlaid on the plot, the direction of the vector showed that gas production was correlated with *M. elsdenii* 4415, but that there were also other taxa likely responsible for gas production during fermentation, especially in the Me− microbiomes.

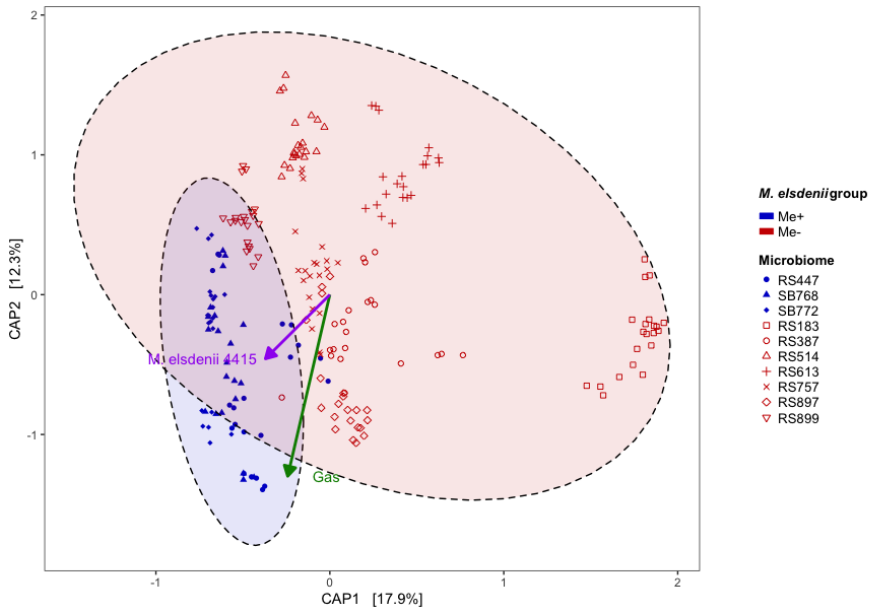

Figure 2.5. Constrained analysis of principal coordinates (CAP) biplot based on Bray-Curtis distance among samples (R2=0.126, p<0.01). Eigenvector for *M. eldenii* 4415 as well as a vector for gas production calculated by correlating gas production with CAP scores for all samples are plotted.

2.3.2 Gut bacteria associated with gas production.

MaAsLin2 was used to identify ASVs that were associated with gas production during fermentation of red kidney beans and sweet potatoes. Five ASVs were significantly associated with gas production (Fig. 2.6A). Among the five ASVs, *M. elsdenii* 4415 had the strongest association with gas production after 48 h of fermentation. The examination of the scatter plot of gas production versus adjusted *M. elsdenii* 4415 relative abundances confirmed the strong correlation between *M. elsdenii* 4415 and gas production (Fig. 2.6B).

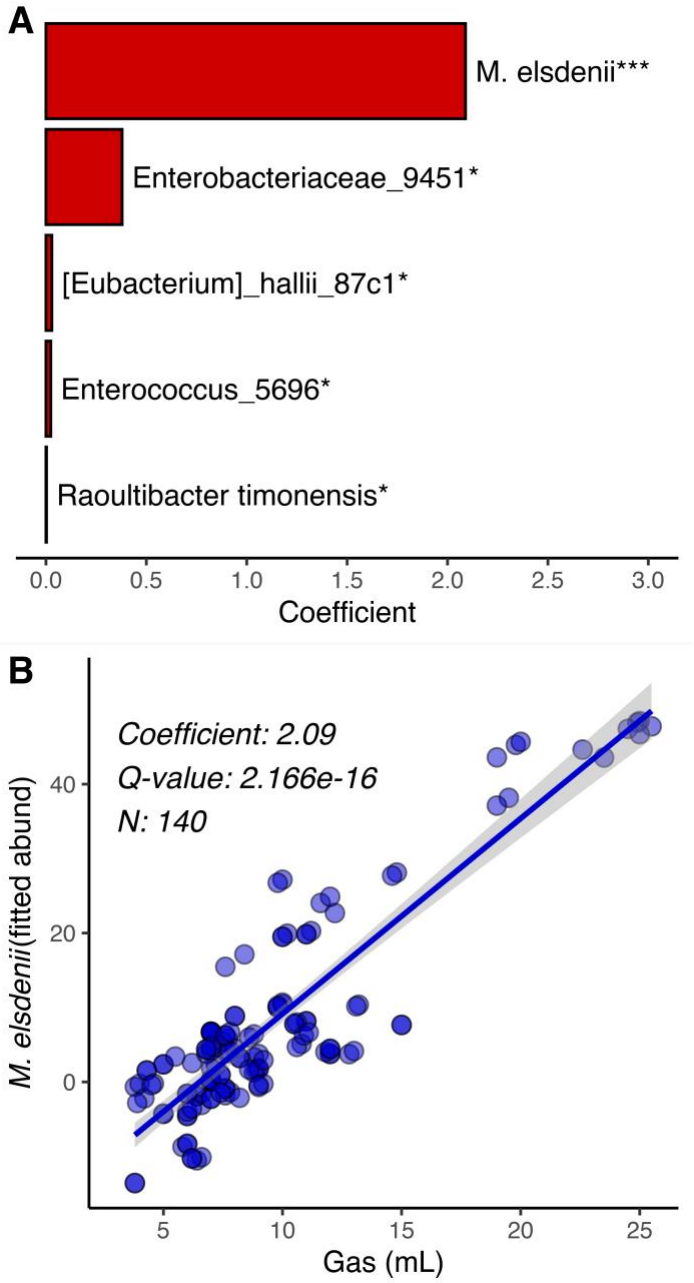

Figure 2.6. MaAsLin2 analysis of ASVs significantly positively associated with gas production (A). Scatter plot of the correlation between *M. elsdenii* and gas production (B). * $P < 0.05$, ***$p<0.001$.

2.3.3 Gut metabolites by microbiomes

After 48 h of fermentation, the Me+ microbiomes resulted in significantly more butyrate production compared to Me− microbiomes, particularly during fermentation of sweet potatoes but also during fermentation of kidney beans (Fig. 2.7C). In contrast, the Me− microbiomes resulted in higher acetate and propionate production compared to Me+ microbiomes after 48 of fermentation of both sweet potatoes and red kidney beans (Fig. 2.7A, B). Within the Me+ microbiomes, it was apparent that a decrease in acetate led to an increase in butyrate production. This suggests that there might be a cross-feeding of acetate by microbiomes to produce butyrate.

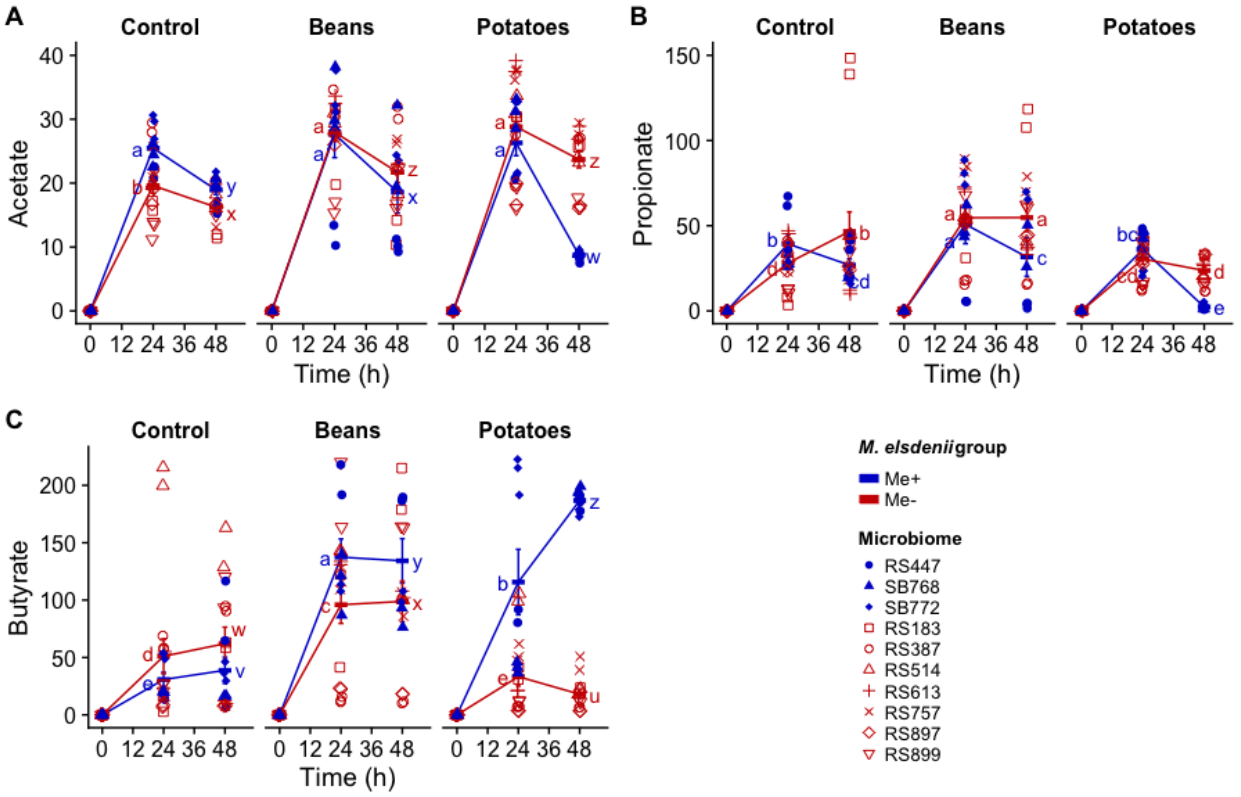

Figure 2.7. Acetate (A), propionate (B), and butyrate (C) production during 48 h of fermentation of kidney beans and sweet potatoes by microbiomes positive for *M. elsdenii* (Me+) and microbiomes negative for *M. elsdenii* (Me-). Error bar shows standard error; different letters denote significant differences among substrates and *M. elsdenii* 4415 groups at the same time point (Tukey's HSD $p<0.05$).

2.4 Discussion

Flatulence is still a sufficient deterrent for some people to consume dietary fiber-containing foods because of the association of these foods with increased gas production (Szczebyło et al., 2020). Consuming foods high in dietary fiber is not the only factor that induces gas symptoms; the gut microbiota is also responsible for differentiating gas production (Manichanh et al., 2014b). In this study Me+ microbiomes produced significantly more gas compared to Me− microbiomes during fermentation of sweet potatoes and red kidney beans. This is consistent with previous studies where *Megasphaera elsdenii* was significantly associated with gas production during the fermentation of pulses using human gut microbiota (Rose et al., 2021), grains using rumen microbiota (Meissner et al., 2014; Sedighi & Alipour, 2019) and purified fibers using horse microbiota (Douthit et al., 2019a).

While Me+ microbiomes had detectable *M. elsdenii* 4415 in fecal samples, which increased dramatically during fermentation, Me− microbiomes also showed a low abundance of *M. elsdenii* 4415 after fermentation even though it was not detected in the fecal inoculum (at 0h of fermentation). Thus, the high gas production contributed by *M. elsdenii* 4415 appears to be dependent on abundance, with sufficiently high relative abundance in stool samples (i.e., detectable) required to manifest the high gas production phenotype during fermentation. Given these findings, *M. elsdenii* 4415 is an important commensal member of the microbiome that is involved in elevated gas production during fermentation of gas-generating foods; but it is not fulfilling all the requirements to be a keystone species. Keystone species are defined as “taxa that individually or in a guild exert a considerable influence on microbiome structure and functioning irrespective of

their abundance across space and time" (Banerjee et al., 2018). *M. elsdenii* 4415 fits the first part of the keystone species definition because it affects gas production strongly, but it fails to meet all the requirements since the abundance was taken into account in this study.

Although our primary focus was on the comparison of gas production between Me+ and Me− microbiomes during fermentation, there were some clear differences between red kidney beans and sweet potatoes in terms of gas production. Surprisingly, Me+ microbiomes that were treated with digested sweet potatoes produced more gas than those treated with kidney beans. Most research on causative factors of flatulence has concentrated on legumes, particularly kidney beans, and has indicated that the oligosaccharides of the raffinose family (raffinose, stachyose, and verbascose) are the main gas-producing factors in kidney beans (Fleming, 1981; Murphy, Horsley, & Burr, 1972). However, relatively low quantities of these oligosaccharides are present in sweet potatoes compared to kidney beans (Den et al., 1986). Thus, it seems unlikely that the high gas production during fermentation of sweet potatoes by the Me+ microbiomes was due to raffinose oligosaccharides. Sweet potatoes have a high content of non-digestible polysaccharides such as cellulose, hemicellulose (xylans, xyloglucans, mannans, arabinogalactans, glucomannans), lignin, pectin, inulin, and resistant starch, which have been suggested to influence gas production (Den et al., 1986; Mei, Mu, & Han, 2010; Palmer, 1982; J. L. Slavin, 2008; Tsou & Yang, 1984).

The findings of the differences in short chain fatty acid production, particularly butyrate and acetate suggest that gas production during fermentation of sweet potatoes and kidney beans may be indirectly caused by fermentation of the non-digestible

carbohydrates through cross-feeding. After 48 h of fermentation, Me+ microbiomes resulted in a significant increase in butyrate production and a decrease in acetate compared to Me− microbiomes, especially during the fermentation of sweet potatoes. Thus, there may be metabolic cross-feeding between species that can ferment dietary fibers to acetate (or lactate) and gas-producing species that convert these acids to butyrate and gas (Kanauchi et al., 1999; Le Blay, Michel, Blottière, & Cherbut, 1999). Indeed, *M. elsdenii* efficiently converts lactate and acetate to butyrate and gas (Counotte, Prins, Janssen, & De Bie, 1981; Forsberg, 1978; Hino, Miyazaki, & Kuroda, 1991). Coincidently, in a recent study that is in accordance with our findings, cross-feeding interactions were observed between bacterial strains that fermented inulin-type fructans (ITF) to acetate and lactate and butyrate-producing bacteria that consumed these acids to produce butyrate and gas, but were not able to metabolize ITF themselves (Moens, Verce, & De Vuyst, 2017).

Although correlation analysis showed that *M. elsdenii* and gas production were strongly correlated, there were four other ASVs from *Eubacterium, Enterococcus, Raoultibacter,* and *Enterobacteriaceae* that were associated with gas production. *Eubacterium* is an abundant genus in human faeces (Leitch, Walker, Duncan, Holtrop, & Flint, 2007). Several studies have reported that some bacterial species from this genus are associated with hydrolytic activities involved in the degradation of insoluble polysaccharides to produce hydrogen gas together with butyrate (Duncan & Flint, 2008; Duncan et al., 2002; Flint, Bayer, Rincon, Lamed, & White, 2008; Leitch et al., 2007). Other studies have revealed that some bacterial species of Enterobacteriaceae can utilize non-digestible carbohydrates and yield gas as a by-product of fermentation. For instance,

in vitro studies found that, *Escherichia coli* and *Enterobacter cloacae* were capable of metabolizing raffinose and fructooligosaccharides, respectively, and resulted in increased gas production during fermentation (Mao et al., 2015, 2018). Several species belonging to the genus *Enterococcus* have been reported to be fibrolytic microorganisms of the human and animal colon and are involved in the breakdown of the dietary fibers found in the plant cell wall; but also, most can produce H_2 as a byproduct of fiber degradation (Robert & Bernalier-Donadille, 2003; Robert, Del'Homme, & Bernalier-Donadille, 2001; Zhang et al., 2017).

Cross-feeding on acetate (or lactate) may also have contributed to gas production in the Me− group. One ASV from *Eubacterium hallii* was also significantly associated with gas production (although much less than *M elsdenii* 4415). Members of this genus are gas and butyrate producers (Falony et al., 2009; Muñoz-Tamayo et al., 2011). In one *in vitro* study, when *E. hallii* was cocultured with *Bifidobacterium adolescentis* during fermentation of potato starch, *B. adolescentis* was shown to metabolize the substrate and produce lactate and acetate which was later utilized by *E. hallii* to produce butyrate (Belenguer et al., 2006). However, it was reported that bacterial species of Enterobacteriaceae do not cross-feed but rather can metabolize non-digestible carbohydrate and produce gas primarily directly by fermentation of carbohydrates; hence this could be why the Me− microbiomes are not as efficient as gas producers (Hartemink, Van Laere, & Rombouts, 1997; Mao et al., 2015, 2018).

Evidently, cross-feeding was stronger in the Me+ microbiomes because the utilization of acetate was very high, but also accompanied with higher butyrate production and gas. It appeared that possibly Me− microbiomes did not have sufficient

butyrate-producing bacterial species to utilize acetate for butyrate production during fermentation. It has been reported that the rate and ratio of SCFA production depend upon the colonic microflora of an individual (Laurentin & Edwards, 2004). If the microbiome composition of an individual contains high counts of butyrate-producing bacterial species, in the presence of fermentable carbohydrates available for fermentation, the conversion of acetate to butyrate will be quantitatively more significant (Morrison et al., 2006).

In conclusion, *M. elsdenii* may be an important but not a keystone species of the gut microbiota that is involved in elevated gas production during fermentation of flatulence-causing substrates. After 48h of fermentation of sweet potatoes and kidney beans, microbiomes that were positive for *M. elsdenii* (Me+) produced significantly more gas than microbiomes that were negative for *M. elsdenii* (Me−). However, Me+ microbiomes that were treated with digested sweet potatoes produced more gas than those treated with kidney beans. Although, *M. elsdenii* was associated with high gas production during fermentation of sweet potatoes and kidney beans other taxa were also associated with gas production and may have contributed to differential gas production among microbiomes and substrates. It was also clear that the high gas production during fermentation was likely a result of cross-feeding on acetate as it was manifested in Me+ microbiomes. This study establishes that microbiome composition has a dramatic influence on gas production during fermentation (even on identical substrates) and that *M. elsdenii* is one commensal member of the microbiome that may be responsible for high gas production during fermentation of flatulogenic foods. Furthermore, in this study

gas production was primarily generated through cross-feeding on acetate (and possibly lactate) and not produced directly from metabolism of non-digestible carbohydrate.

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CHAPTER 3 GAS PRODUCTION IS INDIRECTLY RELATED TO RAFFINOSE DEGRADATION DURING FERMENTATION BY HUMAN GUT BACTERIA

ABSTRACT

Raffinose**,** a compound abundantly found in pulses, is known to cause increased flatulence, which is a significant obstacle to pulse consumption. Therefore, the objective of this study was to determine the relationship between gas production and raffinose degradation among varying microbiomes. The variation in raffinose degradation and gas production among 21 microbiomes was determined, and the variation was associated to the microbiome composition and short-chain fatty acid production. After 24 h of fermentation, fermentation outcomes varied among microbiomes. Unexpectedly, raffinose utilization was not positively but negatively correlated with gas production ($r = -0.44$, $p = 0.047$). Raffinose utilization was also highly significantly positively correlated with acetate production ($r=0.7$, $p < 001$). On the contrary, gas production was strongly positively correlated with butyrate production ($r = 0.78$, $p < 001$). There were several taxa that explained the variation of fermentation outcomes among microbiomes. Taxa from *Bifidobacterium* and *Blautia* were strongly associated with raffinose degradation and acetate production. Several other taxa from *Megasphaera, Anaerostipes, Faecalibacterium,* and *Collinsela*, were significantly associated with gas and butyrate production. This study suggested that gas was not produced directly from the metabolism of raffinose, but rather through cross-feeding between raffinose-degrading, acetate-producing bacteria and acetate-utilizing, butyrate-producing bacteria. Thus, the volume of

gas produced depended on the abundance of bacteria capable of utilizing acetate for butyrate formation and gas production.

3.1 Introduction

Much evidence supports the health benefits associated with the consumption of plant-based diets, particularly pulses (Leterme, 2002; Tharanathan & Mahadevamma, 2003). Increasing intake of pulses is associated with significantly lower blood cholesterol, blood pressure, and the incidence of breast cancer (Abeysekara, Chilibeck, Vatanparast, & Zello, 2012; Hu, 2003; Orlich & Fraser, 2014).

However, one of the obstacles to increased intake of pulses is flatulence, which is widely recognized by consumers as an undesirable side-effect of pulse consumption (Veenstra et al., 2010). This has been attributed to the relatively high concentrations of raffinose family of oligosaccharides (RFOs) in pulses, which are the compounds known to cause excessive gas in humans (Naczk, Amarowicz, & Shahidi, 1997).

RFOs include raffinose, stachyose, and verbascose, which are composed of one, two, or three α-galactosyl residues attached to the fructosyl moiety of sucrose, respectively, via (1→6)-glycosidic linkages (Van den Ende, 2013). These RFOs are not digested or absorbed in the human intestinal tract due to the lack of α-galactosidases to hydrolyze these oligosaccharides into monomers (Kalantar-Zadeh, Berean, Burgell, Muir, & Gibson, 2019). Therefore, RFOs are hydrolyzed and fermented by colonic bacteria for energy, producing gases (hydrogen, carbon dioxide, and methane) and other metabolites such as short chain fatty acids (SCFAs) (Price, Lewis, & Fenwick, 1988). Consequently, some people may be hesitant to increase pulses and other plant foods high in RFOs in their diet due to the fear of excessive flatulence.

In order to improve the acceptability of pulses, several studies have examined different food processing techniques to remove RFOs from pulses, which can presumably

reduce gas production. Overnight soaking followed by pressure cooking has been a traditional practice used for home preparation of pulses (especially dry beans) to reduce RFOs. Other processing techniques to reduce RFO content in pulses are germination, enzyme treatments, autoclaving, and irradiation (Thirunathan & Manickavasagan, 2019). Although these processing strategies may reduce RFOs, several studies emphasized that these strategies do not completely eliminate RFO content in pulses. Rather, the reduction in the levels of RFOs varies from 15%-90% depending on how these techniques are manipulated and the type of pulse (Abdel-Gawad, 1993; Matella et al., 2005; Mulimani & Devendra, 1998; Trugo, Ramos, Trugo, & Souza, 1990). Furthermore, some studies suggest that the removal of RFOs caused no change in total flatus volume (Doris Howes Calloway & Murphy, 1968; Murphy, Horsley, & Burr, 1972).

Additionally, there is evidence that food processing techniques and other strategies designed to remove RFOs from pulses may also reduce the abundance of beneficial gut bacteria (e.g., bifidobacteria and lactobacilli) that offer numerous health benefits to the host (Holscher, 2017). These gut bacteria metabolize oligosaccharides and produce metabolites such as SCFAs (acetate, propionate, and butyrate) that provide health benefits such as protecting the host against pathogens and regulating endocrine, metabolic, and immune functions (Silva, Bernardi, & Frozza, 2020).

Although increased flatulence is an expected outcome among some people after the inclusion of pulses in their diet, several studies have reported that even on the same diet, individuals vary in their response in terms of intestinal gas production (Perera, Russo, Takata, & Bobe, 2020; Tomlin, Lowis, & Read, 1991). This variability may be due to the differences in the types of gut microflora within and between individuals

(Calloway & Burroughs, 1969). One study demonstrated that the differences in microbial composition and stability of the gut microbial ecosystem within people might explain changes in gas production when feed a high flatulogenic diet (Manichanh et al., 2014). However, further research on this topic is still needed.

While previous research has indicated that RFOs may contribute to gas production by the microbiome, no research has investigated whether the heterogeneity in the composition of gut microbiota could affect the volume of gas produced during the fermentation of raffinose. We hypothesized that gas production and raffinose degradation among varying microbiomes would differ and that some taxa would be responsible for high raffinose degradation and associated with high gas production. To test this hypothesis, we determined the variation in raffinose degradation and gas production among 21 microbiomes and associated the variation to microbiome composition and SCFA production.

3.2 Materials and Methods

3.2.1 Fecal sample collection

Fecal samples and written consent forms from 21 healthy adults with no history of gastrointestinal diseases were collected. All procedures involving human subjects were approved by the Institutional Review Board of the University of Nebraska before initiating the study (20210621206P). A fecal slurry was made within 2 hours of defecation by mixing each fecal sample separately with anaerobic sterile phosphate-buffered saline, pH 7.0, containing 10% glycerol as a cryoprotectant at 1:9 w/v inside a sterile filter bag (Filtra-Bag, Thomas Scientific, New Jersey). A stomacher was used to homogenize each fecal slurry for 4 min, then the mixture was transferred to an anaerobic

chamber (containing 5% H_2, 5% CO_2, and 90% N_2, Bactron X, Sheldon Manufacturing, Cornelius, OR, USA) and aliquoted in 15 mL polypropylene centrifuge tubes. Fecal slurries were then stored in -80C freezer until further use.

3.2.2 In vitro fermentation

In vitro fermentation was performed as previously described (Yang & Rose, 2014) with some modifications. Briefly, raffinose was dissolved in fermentation medium at a final concentration of 10 g/L. The fermentation medium contained (per L): 2 g peptone (Fisher Scientific, Waltham, MA, USA), 2 g yeast extract (Fisher Scientific, Waltham, MA, USA), 0.5 g bile salt (Oxoid, Cheshire, England), 2 g $NaHCO_3$, 0.1 g NaCl, 0. 5g L-cysteine (Fisher Scientific, Waltham, MA, USA), 2 mL Tween 80 (Fisher Scientific, Waltham, MA, USA), 1 mL Vitamin K solution (10 µL/1 mL dissolved in ethanol; Alfa Aesar, Haverhill, MA, USA), 4 mL of resazurin solution (1 mg/4 mL dissolved in water; Alfa Aesar, Haverhill, MA, USA), 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 1 mL hemin solution (0.015 g hemin dissolved in 3 mL DMSO), and 0.04 g K_2HPO_4. Inside the anaerobic chamber, 9 mL of pre-reduced medium were added to Hungate tubes and inoculated with 1 mL of each fecal slurry. The tubes were then immediately sealed with a rubber stopper and aluminum seal and incubated at 37°C. The gas volume produced during fermentation was measured after 8 h and 24 h by inserting a needle attached to a glass syringe through the septum and reading the gas volume from the graduations on the syringe. After gas measurements, the fermented samples were aliquoted in 1.5 mL centrifuge tubes and stored at −80 °C. All fermentations were performed in triplicate with separate tubes for 0 h, 8 h, and 24 h measurements

3.2.3 Short chain fatty acids

The fermented samples were thawed and centrifuged at 9600*g* for 10 min. The supernatants was then collected and used for SCFA analysis as previously described (Hartzell, Maldonado-Gómez, Hutkins, & Rose, 2013). In short, 0.4 mL of supernatant was vortex mixed with 0.1 mL of 7 mM 2-ethylbutyric acid in 2 M potassium hydroxide, 0.2 mL of 9 M sulfuric acid, and ~ 0.1 g of sodium chloride in a 2 mL screw cap microcentrifuge tube. Diethyl ether (0.5 mL) was then added, and the mixture was inverted several times followed by centrifugation at 13600g for 1 min. The top layer was collected and injected into a gas chromatograph (Clarus 580; PerkinElmer, Waltham, MA, USA) equipped with a capillary column [Nukol; 30 m (l) × 0.25 mm (i.d.) × 0.25 μm (film thickness); Supelco, Bellefonte, PA] and a flame ionization detector. The quantification of SCFA was done by calculating response factors for each short chain fatty acid (acetate, propionate, butyrate, iso-butyrate, and iso-valerate) relative to 2-ethylbutyric acid using injection of pure standards.

3.2.4 Microbiota composition

Bacterial DNA was extracted from bacterial pellets obtained from SCFA analysis using the BioSprint 96 workstation (Qiagen, Germantown, MD), Biosprint 96 One-For-All Vet kit, stool lysis buffer ASL (Qiagen, Germantown, Maryland), and bead beating. The amplicon sequencing of the V4 region of the bacterial 16S rRNA gene was completed using the Illumina MiSeq platform and the MiSeq reagent kit v2 (2 × 250 bp) (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Sequences were demultiplexed and barcodes were removed prior to sequence analysis with QIIME 2 (Bolyen et al., 2019). Sequence quality control, trimming, chimera removal, and denoising were

performed with DADA2 (Callahan et al., 2016). Forward and reverse reads were truncated to 245 and 160 bp, respectively, to maintain sequence qualities above a phred score of 30. Using DADA2, sequences were dereplicated into 100% amplicon sequence variants (ASVs) for exact sequence matching. Taxonomy was assigned using the SILVA database (Quast et al., 2013). Samples were normalized to the median sequence depth of 13241 reads/sample prior to diversity calculations. Then, for statistical analysis, low abundance spurious sequences were filtered by removing taxa with total number of reads of <0.25% in all samples prior to statistical analysis (Reitmeier et al., 2021). Normalization, diversity calculations, and filtering were performed using the phyloseq package in R (version 4.1.3) (McMurdie & Holmes, 2013).

3.2.5 Data analysis

All data were analyzed using R (version 4.1.3) and RStudio (2022.02.3 Build 492) with various packages as described. Correlations were calculated on log2-transformed data using the 'Hmisc' package (Harrell Jr., 2022). Correlations were performed on mean data by microbiome (i.e., three technical replicate measurements were averaged for each microbiome for a total N=21). Sequencing data were processed using the phyloseq package (McMurdie & Holmes, 2013). Multivariate Association with Linear Models 2 (MaAsLin2) was used to identify ASVs that were associated with fermentation outcomes including gas production, raffinose degraded, acetate, propionate, and butyrate production during fermentation (Mallick et al., 2021). The MaAsLin2 model treated each fermentation outcome as fixed effect and microbiome and time as random effects. Then, MaAsLin2 output results were ranked by coefficient and the top 10 ASVs positively associated and the top 10 ASVs negatively associated with each outcome were selected and used to make

a heatmap. In all cases, these ASVs were highly significantly associated with each outcome (q-value < 0.05). Results for all statistical comparisons were visualized using the ComplexHeatmap, ggplot2, and cowplot packages in R (Wickham, 2016; Wilke, 2018; Zuguang Gu, Roland Eils, & Matthias Schlesner, 2016).

3.3 Results

3.3.1 Fermentation outcomes

Because raffinose oligosaccharides are known to cause elevated gas production, 21 microbiomes were treated with raffinose during 24 h of fermentation to primarily determine the relationship between raffinose degradation and gas production. These outcomes were associated with SCFA production and microbiota composition. Raffinose degradation varied among microbiomes from 21% to 80%, but only three microbiomes utilized over 70% of raffinose (RF009, RF017, and RF020) by the end of 24h of fermentation (Fig. 3.1A). In terms of gas production, microbiomes showed variation in gas produced (ranging from 3mL to 17mL), but two microbiomes (RF002 and RF004) stood out because they produced over 14 mL of gas after 24 h of fermentation (Fig. 3.1B). Because these results were not normally distributed, data were log transformed for data analysis.

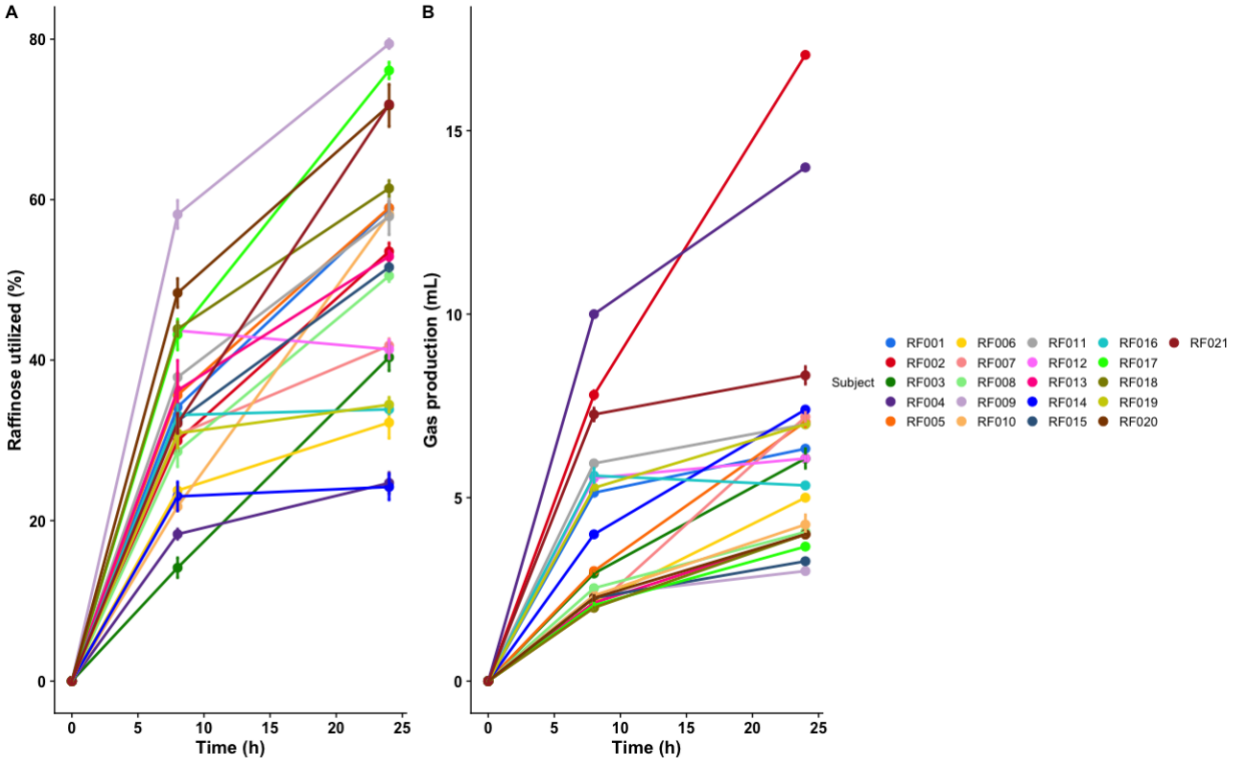

Figure 3.1. Raffinose degradation (A) and gas production (B) by twenty-one microbiomes during 24 h of fermentation of raffinose.

For SCFAs, microbiomes resulted in varied acetate, propionate, and butyrate over 24 h of fermentation (Fig 3.2). However, the same two microbiomes (RF002 and RF004) that produced unusually high gas, resulted in elevated butyrate production compared to the other microbiomes. In addition, microbiome RF005 resulted in exceptionally higher propionate production than the other microbiomes

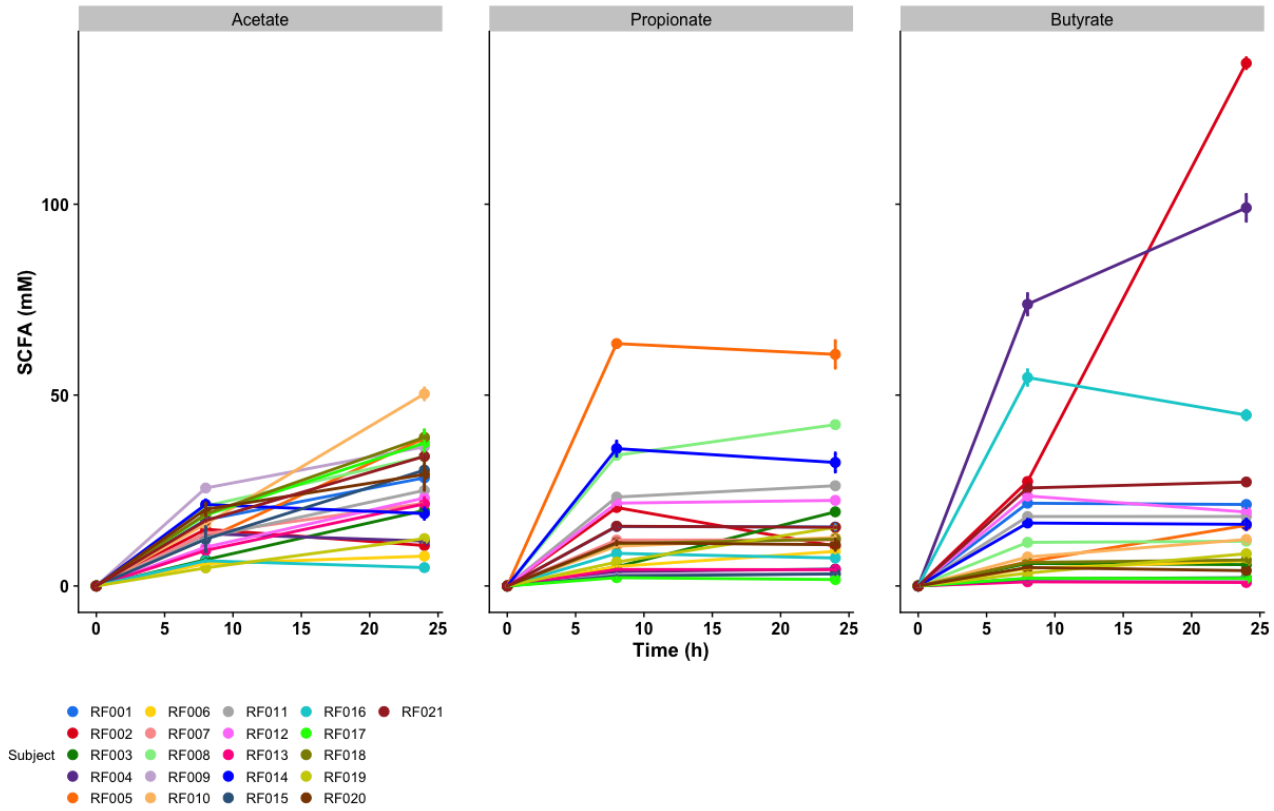

Figure 3.2. Short chain fatty acids production (acetate, propionate, and butyrate) by twenty-one microbiomes during 24 h of fermentation of raffinose.

Unexpectedly, raffinose utilization was not positively but negatively correlated with gas production (Fig. 3.3B). Raffinose utilization was also highly significantly positively correlated with acetate production (Fig 3.3C). Gas production was strongly positively correlated with butyrate production (Fig.3.3 D).

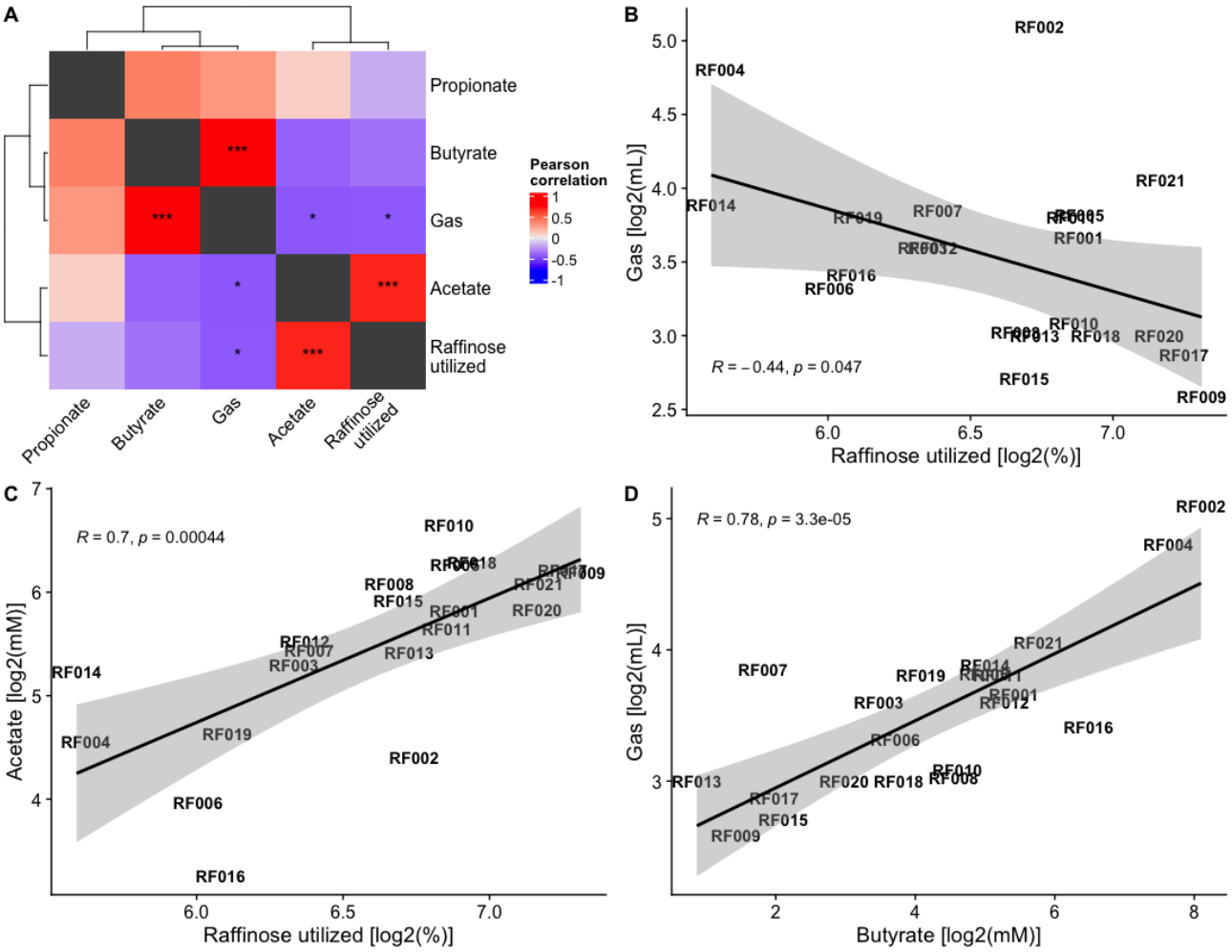

Figure 3.3. Heatmap of Pearson correlations among fermentation outcomes after 24 h of fermentation (A); Scatter plot of fermentation metabiltes that strongly correlated negatively (B) or positively (C, D) with each other. *p<0.05, ***p<0.001)

3.3.2 Association of fermentation outcomes with microbiota composition

To identify taxa associated with fermentation outcomes during raffinose degradation, a MaAsLin2 analysis was performed. This analysis confirmed that raffinose degradation and acetate production are associated, while gas production and butyrate production are tightly linked. Interestingly, two ASVs from *Bifidobacterium* were positively associated with raffinose degradation and acetate production, while also being negatively associated with gas production. There were also several *Blautia* that were

associated with raffinose degradation and/or acetate production. In contrast, members of *Anaerostipes*, *Faecalibacterium*, *Collinsela*, and most notably *Megasphaera* were strongly positively associated with gas and butyrate production (Fig. 3.4).

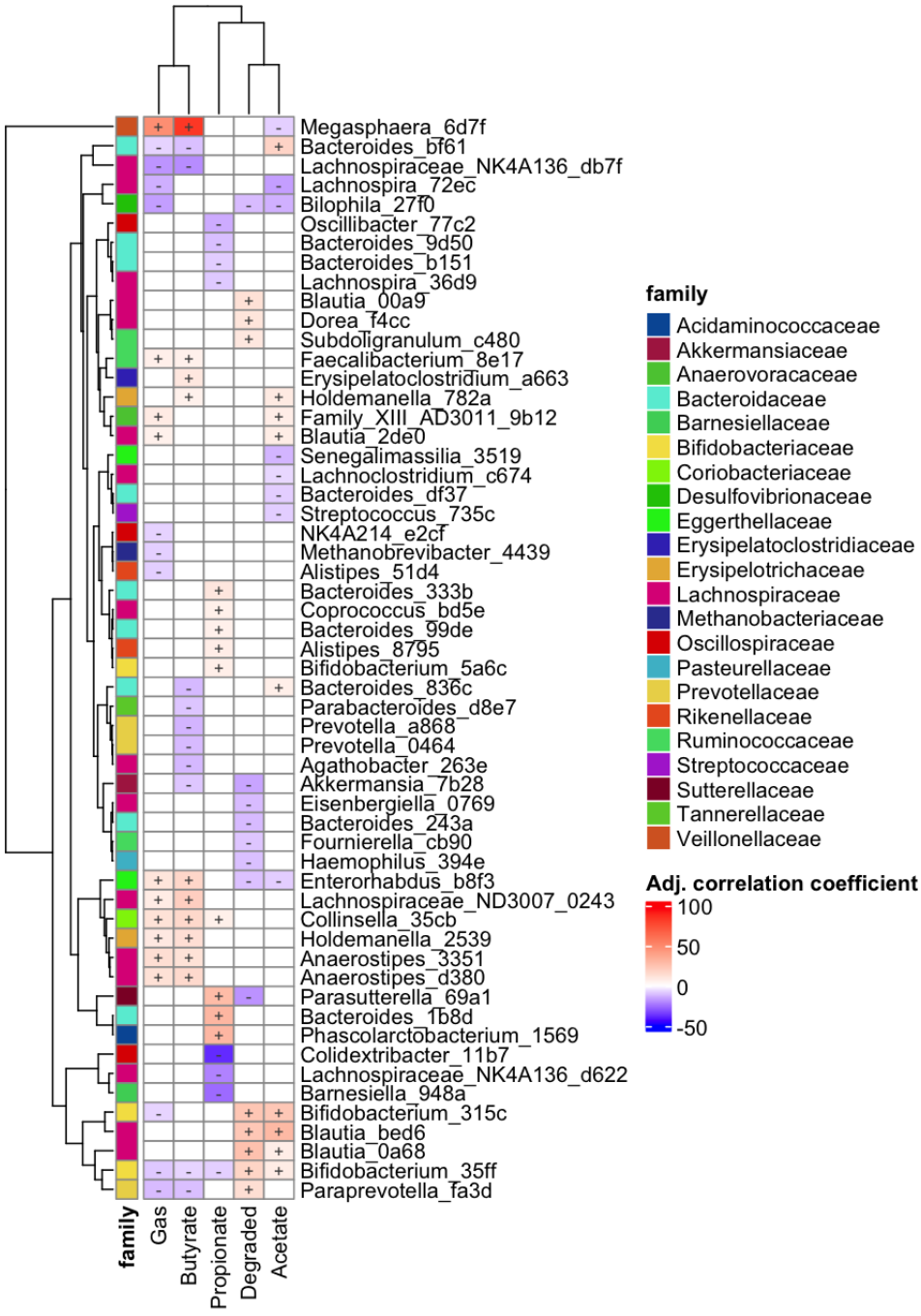

Figure 3.4. Heatmap describing most strong associations between ASVs, and fermentation outcomes detected by MaAsLin2 ($p<0.05$ and $qval<0.25$). Positive associations are colored in red, while negative associations are colored in blue. The effect size (adj. correlation coefficient) was calculated according to this formula: (-log(qval)*sign(coeff)).

3.4 Discussion

Raffinose, a compound abundantly found in pulses is implicated in elevated gas production following pulse consumption, which is a significant obstacle to increased consumption. Therefore, it was hypothesized that gas production and raffinose degradation among varying microbiomes would differ and that some taxa would be responsible for high raffinose degradation and associated with high gas production. To test this hypothesis, the variation in raffinose degradation and gas production among microbiomes was determined and the variation was associated to microbiome composition and SCFA production. Although our experiment supported gas production and raffinose degradation varying among microbiomes, it did not support the association of certain taxa to high raffinose degradation and gas production because those two variables were not directly linked. Instead, analysis of fermentation outcomes demonstrated that raffinose degradation and gas production were negatively correlated with each other. This outcome was unexpected because raffinose degradation and gas were expected to be positively correlated. Recent two studies claim that raffinose utilization and gas production are related, however they did not demonstrate how those two variables are directly correlated (Amorim et al., 2020; Dahl, Hanifi, Zello, & Tyler, 2014). On the other hand, raffinose was associated with with acetate production. It has been reported that acetate is the most abundantly generated SCFA during the fermentation of raffinose and it is strongly associated with raffinose degradation (Amorim et al., 2020; Ose et al., 2018). As expected, gas production was strongly positively associated with butyrate production. Yu, et al. reported gas and butyrate were correlated during the fermentation of dietary carbohydrates and suggested that it would

be unlikely for subrates to yield both low gas and high butyrate production (Yu et al., 2020). So, we then looked for taxa that were associated with raffinose degradation and acetate and also taxa that were associated with butyrate and gas production.

Recent studies have focused on raffinose metabolism by colonic bacteria due to its prebiotic potential, but also flatulence-inducing potential in some hosts (Amorim et al., 2020). Although humans harbor over 1000 bacterial species, only over a hundred species have the ability to utilize raffinose as an energy source (Mao et al., 2018). The findings in this study showed that several *Bifidobacterium* ASVs were the most strongly associated ASVs with raffinose degradation during 24 h of fermentation of raffinose. It was reported that bacterial species that could utilize raffinose were those that demonstrated α-galactosidase and β-fructosidase activity (Chen & Mustapha, 2012; Zartl et al., 2018). Among other colonic bacteria, bifidobacteria species display a preferential oligosaccharide metabolism due to their high α-galactosidase activity, providing them a competitive advantage over other colonic bacteria species that degrade oligosaccharides. Recent studies have demonstrated that the addition of raffinose in the fermentation media stimulated the growth of bifidobacteria species (Mao et al., 2018; Trindade, Abratt, & Reid, 2003; Xiao, Tanaka, Qian, Yamamoto, & Kumagai, 2000). In this study, *Blautia* ASVs, members of *Lachnospiraceae*, were also significantly associated with raffinose utilization during fermentation. Several studies reported that strains of *Blautia* also possess α-galactosidase activity and were found to be capable of utilizing raffinose (Lafond et al., 2020; Liu et al., 2021).

In the context of raffinose degradation, *Bifidobacterium* and *Blautia* were also associated with acetate production during fermentation. Indeed, the principal metabolic

products of raffinose fermentation by *Bifidobacterium* and some *Blautia* species are acetate and lactate (Belenguer et al., 2009; L. De Vuyst, Moens, Selak, Rivière, & Leroy, 2014). These acids are the major intermediate carbohydrate breakdown metabolites because they can serve as carbon and energy sources for cross-feeding bacteria (Belenguer et al., 2009; Duncan, Louis, & Flint, 2004). Several studies have identified acetate and lactate as important precursors of butyrate production in human fecal samples (Bourriaud et al., 2005; Morrison et al., 2006).

Acetate and lactate form the basis for cross-feeding interactions of raffinose degraders, such as *Bifidobacterium* and *Blautia*, with butyrate-producing bacteria that do not utilize raffinose themselves, but can utilize the acetate for metabolism. Although lactate was likely involved in the cross feeding, we did not measure it in this study. Butyrate-producing bacteria metabolize acetate and lactate as carbon and energy sources to produce butyrate and gas (Falony, Vlachou, Verbrugghe, & De Vuyst, 2006). On the contrary, this study showed that *Bifidobacterium* and other raffinose degraders were neither associated with gas nor butyrate production. Recent *in vitro* studies confirmed that *Bifidobacterium,* the main raffinose utilizers, do not produce butyrate and gas (Luc De Vuyst & Leroy, 2011; Falony, Lazidou, et al., 2009; Falony, Verschaeren, et al., 2009).

In this study, members of *Anaerostipes*, *Faecalibacterium*, and *Megasphaera* were significantly associated with butyrate and gas production. *Anaerostipes* and *Megasphaera* can utilize both lactate and acetate to produce butyrate, while *Faecalibacterium* can only utilize acetate (Barcenilla et al., 2000; Belenguer et al., 2009; Duncan, Barcenilla, Stewart, Pryde, & Flint, 2002; Duncan et al., 2004). These bacteria

use butyryl-CoA:acetate CoA transferase as the main metabolic pathway to produce butyrate as illustrated in (Fig. 5) . Pyruvate is the central pivot of butyrate formation. It is oxidized to acetyl coenzyme A (Acetyl-CoA), which is then combined with another Acetyl-CoA to form Acetoacetyl-CoA. Subsequent reduction of Acetoacetyl-CoA yields butyryl-CoA. Then finally, butyryl-CoA: acetate CoA transferase moves the CoA moiety to external acetate, leading to the production of butyrate and acetyl-CoA (Duncan et al., 2002; Louis et al., 2004).

Anaerostipes, *Faecalibacterium*, and *Megasphaera* ASVs have been reported to be involved in cross-feeding with *Bifidobacterium*, yielding increased butyrate and gas production. In a coculture of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* there was enhanced butyrate formation by *F. prausnitzii* in the presence of *B. adolescentis* during the fermentation of different complex carbohydrates (Rios-Covian, Gueimonde, Duncan, Flint, & De Los Reyes-Gavilan, 2015). In other cross-feeding experiments, *B. adolescentis* was shown to metabolize carbohydrates and produce lactate and acetate which was later utilized by members of *Anaerostipes* and *Megasphaera* to produce butyrate and gas (Moens, Verce, & De Vuyst, 2017)(Falony et al., 2006).

In conclusion, gas production and raffinose degradation varied among microbiomes. However, this variation was mainly due to the cross-feeding interactions between the raffinose degrading and butyrate-producing bacteria within a microbiome during fermentation. It was clear that gas production during fermentation was likely a result of cross-feeding on acetate (and most likely lactate) between raffinose degrading, acetate-producing bacteria and acetate utilizing, butyrate-producing bacteria. This study established that gas production during fermentation of raffinose depends on the

abundance of butyrate-producing ASVs capable of utilizing the acetate produced by the primary degraders. Thus, raffinose is only indirectly responsible for gas production by the human gut microbiome.

3.5 References

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