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Fermentation of Prebiotics in Whole Food Powders by Probiotic Lactic-Acid Producing Bacterial Strains to Identify Synbiotic Combinations

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FERMENTATION OF PREBIOTICS IN WHOLE FOOD POWDERS BY PROBIOTIC LACTIC-ACID PRODUCING BACTERIAL STRAINS TO IDENTIFY SYNBIOTIC COMBINATIONS

by

Michaela Brubaker

Thesis submitted in partial fulfillment
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ABSTRACT

Fermentation of Prebiotics in Whole Food Powders by Probiotic Lactic-Acid Producing Bacterial Strains to Identify Synbiotic Combinations

By

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Utah State University, 2019

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Dietary interventions with probiotic lactic acid bacteria (LAB) and prebiotics, complex dietary fibers that promote LAB growth, may favorably shift the gut microbiome to reduce colorectal cancer risk. Our primary hypothesis was that the LAB strains NCFM *Lactobacillus acidophilus* and HN019 *Bifidobacterium lactis* would thrive in the presence of agave, green banana, black raspberry, baobab fruit, or pomegranate peel whole food powders by fermenting their oligosaccharide (OS) components into lactic acid end products. LAB strains were cultured in media with no carbohydrate, purified OS, or one of the whole food powders. LAB strains cultured with agave appeared to grow better compared to the purified OS treatment as determined by the optical density measurement ($p < 0.0001$). Also, the medium pH for *L. acidophilus* cultured with agave or black raspberry decreased significantly more than with purified OS ($p < 0.0001$), indicating a greater rate of fermentation. For *B. lactis* cultured with agave, black raspberry, or baobab fruit the medium pH was not significantly different from the purified OS, suggesting that these foods are fermented at least as well as the purified OS. However, the effect of green banana, black raspberry, or pomegranate peel on bacteria growth could not be determined because the culture medium was too opaque for measurements. The

medium pH of *L. acidophilus* cultured with baobab fruit was not significantly different than the no carbohydrate control. Optical density measurements suggested that baobab fruit did not support growth of *L. acidophilus,* though *B. lactis* grew in the presence of baobab as well as the purified OS. Lastly, neither green banana nor pomegranate peel appeared to be utilized by LAB strains as culture media pH was not changed. In conclusion, results of this study suggest that agave and black raspberry powders support lactic acid bacteria fermentation, while green banana and pomegranate peel did not under the culture conditions employed in this study. Additionally, the fermentation of baobab powder appeared to be dependent on the probiotic strain. Of the bioactive food powders tested, black raspberry appears to be the most promising candidate for future synbiotic colorectal cancer dietary intervention studies.

35 pages

DEDICATION

To my mom for teaching me how to stay organized and to my dad for teaching me how to write.

Thank you both for all of your love and support over the years.

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INTRODUCTION

Americans commonly consume a diet that contains excess fat, simple carbohydrates, and sodium, but that lacks many important micronutrients. In recent years, the link between this Western dietary pattern, inflammatory bowel disease (IBD) and colorectal cancer (CRC) has become increasingly apparent. Approximately 3 million Americans currently suffer from IBD, with a disproportional number of cases occurring in people living in poverty and suburban neighborhoods (Dahlhamer *et al.*, 2016). The number of people suffering from IBD has increased nearly two-fold since 1999. While not all IBD patients develop CRC, the chronic inflammation of the lower gastrointestinal tract associated with IBD puts these patients at an increased risk for developing CRC. The risk of developing CRC increases from 2% after 10 years, to 8% after 20 years, and to 30% after 30 years of living with IBD (Eaden *et al.*, 2001). However, some patients develop CRC without a history of IBD.

According to the American Cancer Society, CRC is a major cause of cancer death in the United States, second only to lung cancer (Siegel *et al.*, 2018). Diet is a major contributing factor for this disease. Several cohort studies have shown an increased risk for developing CRC with the consumption of a Western diet (Meyerhardt *et al.*, 2007). The connection between diet and various cancers was first proposed in 1981. At the time, it was estimated that diet could be responsible for 70% of all cancer cases and 35% of cancer related deaths (Doll and Peto, 1981). Cancer is a major cause of death in most developed countries, especially those that follow a Western dietary pattern. Among Westernized countries, cancer accounts for approximately 25% of deaths (Boyle and Langman, 2000). The increasing consumption of a Western diet helps to explain the increasing number of IBD and CRC cases and related deaths.

Because of the ability of inflammatory molecules to modulate cancer development, inflammation has been proposed as the seventh hallmark of cancer (Mantovani, 2009). Inflammation of the colon and rectum is thought to be a major precursor to CRC as this inflammation facilitates tumorigenesis. Exposure to a chemical irritant, such as dextran sodium sulphate (DSS), in drinking water is commonly used to promote intestinal inflammation in murine models (Wirtz *et al.*, 2007). Administration of DSS can be used to study both acute and chronic colitis. Additionally, the DSS-induced colitis model has been shown to cause dysbiosis of the gut microbiome (De Fazio *et al.,* 2014). As discussed later, the gut microbiome plays an important role in both IBD and CRC. CRC is often studied using a colitis-associated cancer model, a two-hit model that incorporates exposure to a chemical carcinogen to initiate mutations in the colon tissues in combination with exposure to DSS which promotes inflammation of the colon epithelium (Clapper *et al.*, 2007). The increased tumor incidence associated with this model makes it more feasible to study CRC than by inducing inflammation alone. The link between inflammation and CRC is an important aspect of prevention and treatment of this disease.

The composition of the gut microbiome is another important aspect of both IBD and CRC. The possibility of a connection between CRC and the gut microbiota was first made in 1975 (Gagnière *et al.*, 2016). Since then, numerous studies have sought to understand the relationship between the gut microflora and the disease pathway leading to CRC. In a healthy person, the gut microbiome is composed largely of bacteria belonging to phyla *Firmicutes* or *Bacteroidetes* (Eckburg *et al.*, 2005). However, in patients suffering from IBD and CRC, the composition of the gut microbiome is markedly changed. A relative decrease in *Firmicutes* and an increase in *Bacteroidetes*, along with a marked decrease in species diversity has been

observed in individuals with IBD (Walker *et al.*, 2011). An increase in pathogenic bacteria and a decrease in mutualistic bacteria are also thought to play an important role in the development of these diseases. Of the most importance to this project, CRC is associated with decreases in the genera *Lactobacillus* and *Bifidobacterium* (Gagnière *et al.*, 2016). These mutualistic bacteria play an important role in protecting the gastrointestinal tract from inflammatory diseases. They help to "accelerate colon epithelial cell turnover," which inhibits pathogens (Feng *et al.*, 2015). Because of the importance of the gut microbiota to IBD and CRC, altering the intestinal bacteria composition offers the possibility to reduce severity of symptoms, and plays a crucial role the prevention of these diseases.

Modification of the gut microbiome can be achieved through the administration of probiotics. According to the Food and Agriculture Organization of the United Nations and the World Health Organization, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO and WHO, 2006). Probiotics are commonly sold commercially combined with milk products or in capsules. *Lactobacillus* and *Bifidobacterium* are some of the most commonly used bacteria genera in commercial probiotic products (McGarr *et al.*, 2005). The use of probiotics has been shown to be useful in reducing the effects of IBD and CRC. Probiotics improve general intestinal health and reduce inflammation (Ménard *et al*, 2004). Because of the common use of *Lactobacillus* and *Bifidobacterium* as commercial probiotics and their shown health benefits, the species *Lactobacillus acidophilus* (strain NCFM) and *Bifidobacterium lactis* (strain HN019) were selected for use in this project.

NCFM is a gram-positive rod bacterium that lacks catalase activity (Sanders and Klaenhammer, 2001). Catalase protects organisms from oxidative damage that occurs in aerobic environments. Because NCFM lacks catalase activity, it grows best in anaerobic environments,

such as the gastrointestinal tract of mammals. NCFM has long been used as a commercial probiotic. Originally isolated from a human source in the 1970s, this bacteria strain has now been on the market for over 40 years (Sanders and Klaenhammer, 2001). Additionally, the benefits of NCFM when taken as a probiotic has been studied extensively. Like NCFM, HN019 is also a gram-positive rod bacterium that lacks catalase activity (Sanders, 2006). The similarities between NCFM and HN019 help to explain their common use as probiotics. HN019 was originally isolated from yogurt and has since been sold commercially in many different products (Sanders, 2006). HN019 has also been widely studied over the years. Importantly, this bacterium strain has been shown to survive transit to the lower gastrointestinal tract and has also been shown to cause increases in both *Bifidobacterium* and *Lactobacillus* species in the gut (Sanders, 2006). The wide use of these bacteria species as commercial probiotics, along with the large amount of research done on their physiological effects in the digestive system made them the ideal candidates for this project, as well as for future colorectal cancer dietary intervention studies.

Prebiotics are components of the diet that cannot be digested by the host but improve health by increasing the growth and activity of beneficial bacterial species (Diplock *et al.*, 1991). Unlike probiotics, prebiotics modulate the gut microbiome without the addition of exogenous bacteria and only affect species that are already present. Oligosaccharides (OS) (**Figure 1**), which are a type of complex dietary fiber, are often used as prebiotics. Fructo-oligosaccharides (FOS) are linear chains of fructose ranging from 3 to 60 monosaccharides in length (Sabater-Molina *et al.*, 2009) (**Figure 1**). FOS are one of the most common types of OS found in the human diet and are found naturally in a variety of fruits and vegetables. FOS stimulate the growth of beneficial bacteria species (Cruz-Cárdenas *et al.*, 2015). The ability of bacterial

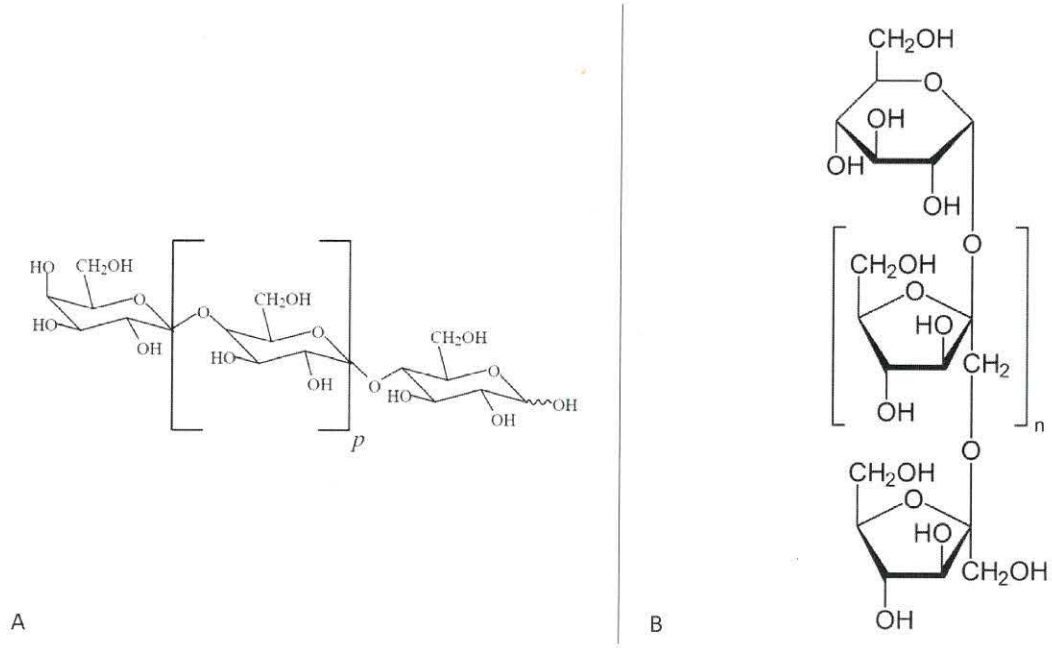

Figure 1. Example oligosaccharide structures. A) An example oligosaccharide structure, with 3-60 monosaccharides linked to form a chain and **B)** inulin, an example fructo-oligosaccharides, with fructose monomers linked together.

species to use OS is what makes them useful prebiotics. Both *Bifidobacterium* and *Lactobacillus* can ferment FOS because of their ability to produce β-fructofurandosidase (Castro-Zavala *et al.*, 2015). In addition to promoting the growth of beneficial bacteria, prebiotics also inhibit the growth of pathogenic species. Both FOS and inulin reduce the amounts of the pathogenic bacteria *Escherichia coli* and *Clostridium* species (Wang and Gibson, 1993). *In vivo*, the effects of prebiotic supplementation result in changes in a relatively short time, and only last as long as supplementation is continued. In one dietary intervention study, the substitution of FOS or inulin for sucrose significantly altered the gut microbiome in only 15 days (Gibson *et al.*, 1995). Prebiotic supplementation not only impacts the microbiome, but also has effects on overall gut health. For example, inulin supplementation was found to reduce the expression of serum and jejunal proinflammatory cytokines, suggesting that this intervention may prevent chronic

inflammation that precedes IBD and CRC (Hijová *et al.*, 2013). Because prebiotics increase growth and activity of beneficial bacteria, they can be used to increase the effectiveness of probiotics. This concept is described using the term "synbiotic", meaning combined use of prebiotics with probiotics to increase the survival of probiotic bacteria for greater health outcomes (Diplock *et al.*, 1991). For this project, we explored this notion of synbiosis while addressing the question of whether providing a fermentable material in the form of fructooligosaccharides would sustain or promote growth of beneficial bacteria strains NCFM or HN019.

The agave plant (**Figure 2**), which is native of Central America, is well known for its uses in the production of alcohol beverages and sugar-substitutes. However, agave also has the highest known OS content of any food item (Nava-Cruz *et al.*, 2014), thus making agave an excellent source of prebiotics. Additionally, agave is rich in a variety of other bioactive molecules including tannins, flavonoids, and saponins (Nava-Cruz *et al.*, 2014). These molecules can have a variety of beneficial impacts on health including having antioxidant and anti-inflammatory properties. One saponin isolated from agave has been shown to inhibit the first

Figure 2. Whole foods used as sources of oligosaccharides for testing as possible prebiotics. A) agave, *Agave tequilana*; **B)** green banana, *Musa acuminate*; **C)** black raspberry, *Rubus occidentalis*; **D)** baobab fruit, *Adansonia digitata*; and **E)** pomegranate peel, *Punica granatum* L.

stage of inflammation (da Silva *et al.*, 2014). The anti-inflammatory and antioxidant properties of these molecules help prevent chronic disease, especially cancer. Agave also has antibacterial properties against a variety of pathogenic bacteria species (Nava-Cruz *et al.*, 2014). Because of the link between pathogenic bacteria and the development of IBD and CRC, agave has the potential to help reduce the risk for these diseases. Additionally, NCFM and HN019 can ferment agave fructans. Compared to chicory, another common source of inulin, agave had higher levels of fermentation and provided a more constant dose-dependent response (Castro-Zavala *et al.*, 2015). Because of the high OS content and known fermentation potential in NCFM and HN019, agave made an excellent experimental FOS source to test the experimental protocol design outlined below as a positive control. Further, the agave fermentation results allowed for comparison with other whole food FOS sources to determine the relative amounts of OS in these foods.

Bananas are the most commonly consumed fresh fruit in the United States, with the average American consuming 11.4 pounds of bananas a year (ERS, 2015). Bananas are a global food staple and are the fifth most economically important food crop worldwide (Singh *et al.*, 2016). Bananas come in many different species and cultivars, and consumption varies based on location. In the United States, the AAA triploid cultivars of *Musa acuminate,* called Cavendish bananas, are the most commonly consumed type (Singh *et al.*, 2016). While bananas are a common source of FOS, the exact FOS content varies greatly between varieties. Ripe Cavendish bananas have the second highest inulin content compared to ten other banana cultivars (Cruz-Cárdenas *et al.*, 2015). Bananas also contain several different bioactive molecules. Most importantly they contain phenolic compounds, caretonoids, and flavonoids (Singh *et al.*, 2016). Like the bioactive molecules in agave, these compounds also act as antioxidants and have anti-

inflammatory properties. Bananas have a higher antioxidant capacity than some of the other berries, and because many of the phenolic compounds found in bananas bind to the cell wall, the antioxidants are more bioavailable for absorption (Singh *et al.*, 2016). Additionally, ripe Cavendish banana fiber has been shown to be fermented by NCFM and HN019 (do Espírito Santo *et al.*, 2011). Unripened bananas have a lower sugar content compared to ripened bananas, as starch is converted to sugar during the ripening process (Pua and Davey, 2007). The use of green unripened Cavendish bananas (**Figure 2**) instead of the ripened fruit reduces the glycemic index of the diet. However, the fermentation potential of green unripened bananas has not been determined. Because bananas contain several bioactive molecules, are a common source of FOS, and are an extremely popular fruit, they make ideal candidates for future CRC dietary intervention studies.

Black raspberries resemble closely related red raspberries but are darker in color (**Figure 2**). The domesticated specie of black raspberry, *Rubus occidentalis*, is native to eastern North America (Kula and Krauze-Baranowska, 2016). While significantly less popular than red raspberries, the demand for black raspberries has grown in recent years. The increased knowledge of the bioactive molecule content of black raspberries is largely responsible for this rise in popularity. Black raspberries have been found to contain significantly higher amounts of bioactive molecules compared to red raspberries, with nearly 3, 6, and 10 times higher phenolic compound, flavonoid, and anthocyanin content respectively (Gansch *et al.*, 2009). This combination of bioactive molecules results in an overall higher antioxidant capacity, at least ten times higher than most other fruits and vegetables (Gansch *et al.*, 2009). The antioxidant and anti-inflammatory properties of black raspberries have been shown to help prevent and reduce severity of colorectal cancer. In pre-clinical mouse models, supplementation with black

raspberry powder significantly decreased colon tumor number and size (Pan *et al.*, 2015 and 2018). Further, the effects of black raspberry supplementation are beginning to be studied in humans. One pilot study found positive changes in tissue biomarkers when colorectal cancer patients were supplemented with black raspberries (Wang *et al.*, 2011). The gut microbiota plays an important role in how black raspberries have these effects. The use of antibiotics to eliminate the gut microbiota counteracted the effects of black raspberry supplementation on colorectal cancer outcomes (Pan *et al.*, 2018). The specific effects black raspberries have on the gut microbiome still need to be determined. Because black raspberries are used extensively in colorectal cancer dietary intervention studies they make an excellent prebiotic to test in this study. Additionally, the ability of lactic acid producing bacterial strains to ferment black raspberries will help to determine the relative oligosaccharide content of these fruits.

The African baobab tree, *Adansonia digitata*, has been used in traditional medicines thousands of years. The leaves, seeds, and fruit pulp have long been used because of their anti-inflammatory and antioxidant properties (Rahul *et al.*, 2015). Only recently have these properties gained baobab attention as a 'superfood' in Western society. The baobab fruit (**Figure 2**) is of the most interest as the fruit pulp has a higher overall antioxidant capacity compared to the leaves (Vertuani *et al.*, 2002). Additionally, the baobab fruit has a higher antioxidant capacity than many commonly consumed fruits. The vitamin C content is particularly high, being almost ten times higher than in oranges (Rahul *et al.*, 2015). In addition to antioxidants, baobab contains several other bioactive molecules. Baobab has also been found to contain high amounts of polyphenols (Garvey *et al.*, 2017), and flavonoids (Braca *et al.*, 2018). Furthermore, baobab fruit is rich in certain micronutrients. The fruit pulp is extremely calcium rich and contains relatively high levels of potassium and magnesium (Osman, 2004). The macronutrient content of baobab

fruit also makes it a promising functional food. Baobab fruit is low in fat and sugar but is has an extremely high fiber content (Braca *et al.*, 2018). Because baobab is a relatively novel food in Western society, its effects on colorectal cancer and the gut microbiome has not yet been evaluated. However, baobab has been shown to improve satiety, likely due to its high fiber content (Garvey *et al.*, 2017). Of most interest to this study is the dietary fiber content of the baobab fruit. Baobab has been found to be particularly rich in pectin (Rahul *et al.*, 2015). The known oligosaccharide content and strong antioxidant capacity of baobab makes it a promising prebiotic. The fermentation potential of baobab by lactic acid bacterial strains is not known.

The pomegranate, *Punica granatum* L., is native to the Middle East and was one of the earliest fruits to be cultivated (Durgaç *et al.,* 2008). Pomegranates are now grown throughout the world and thrive in Mediterranean climates. Annually, approximately 1.5 million tons of pomegranates are produced worldwide (Fischer *et al.,* 2011). Pomegranates have grown in popularity due to the health benefits associated with their juice and arils. The pomegranate peel (**Figure 2**) is considered a waste byproduct of the juicing process and is being tested for use as a livestock feed supplement (Akhtar *et al.,* 2015). However, recent studies have shown the pomegranate peel to contain higher amounts of bioactive molecules compared to the rest of the fruit. Pomegranate peel extract was found to have a 10 times higher phenolic content, along with higher levels of flavonoids and anthocyanins, and a higher antioxidant capacity compared pomegranate pulp extract (Li *et al.,* 2006). However, it is important to note that differences have been found between cultivars grown in different regions. As expected, cultivars with darker peel color contained higher levels of phenolic compounds (Gözlekçi *et al.,* 2011). Because of its high antioxidant capacity, pomegranate peel extract is now being used as a dietary supplement. Pomegranate peel supplements are often sold commercially as capsules or tablets because of low

palatability (Akhtar *et al.,* 2015). Pomegranate peel also contains higher levels of fiber, making it a promising prebiotic. The dietary fiber content of pomegranate peel has been found to be as high as 62% (Hasnaoui *et al.,* 2014). Little research has been done on the effects of pomegranate as a prebiotic. Pomegranate byproduct (the entire fruit with the juice removed) was not found to significantly effect the growth of *B. lactis*, while it negatively effected the growth of multiple pathogenic bacterial species (Bialonska *et al.,* 2009). The effects on other probiotic bacterial strains was strain specific. Another study found pomegranate peel extract to help preserve probiotic bacterial strains in functional ice cream (Sagdic *et al.,* 2011). However, the effect of pomegranate peel alone has not been tested. The high antioxidant and fiber content of pomegranate peel gives it promise as a functional food and prebiotic. The utilization of pomegranate peel also serves to reduce the impact of increasing pomegranate production.

The aims of this study were to determine the ability of NCFM and HN019 to ferment agave, green banana, black raspberry, baobab fruit, or pomegranate peel whole food powders, and to use these results to determine the relative amounts of OS contained in these foods. The main objective was to determine which of these pre- and pro-biotic combinations are best suited for future synbiotic CRC dietary intervention studies.

MATERIALS AND METHODS

Bacterial Strains

Probiotic bacterial strains were generously donated by Danisco (DuPont USA, New Century, KS). The minimum colony forming units (CFUs) per gram where provided with samples. *L. acidophilus* NCFM contained at least 200 billion CFUs per gram, while *B. lactis* HN019 contained at least 300 billion CFUs per gram. Bacteria powders were stored at -20 °C until use.

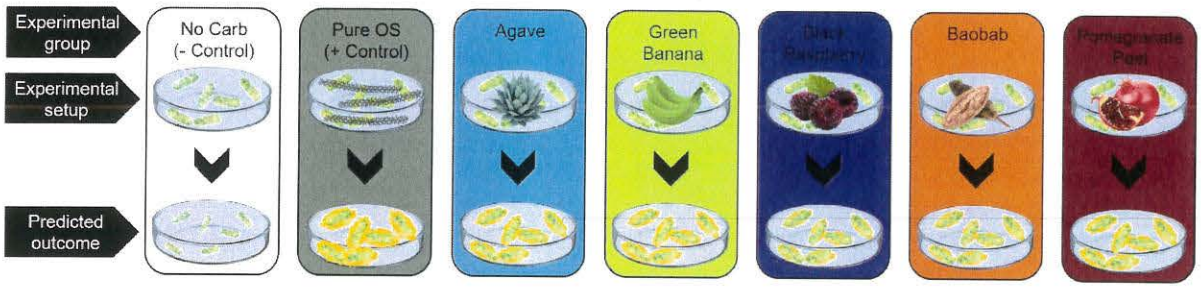

Figure 3. General study design and expected results. Whole food sources of agave, green banana, black raspberry, baobab, and pomegranate peel were used as carbohydrate source to test prebiotic potential compared to a no carbohydrate negative control and a purified OS positive control. Fermentation was expected with every carbohydrate source except for negative control.

Media Constituents

Media constituents (**Figure 3**) were obtained from a variety of commercial sources. Lactobacilli MRS broth without dextrose (Alpha Biosciences, Baltimore, MD) was used as the base for all media. Carbohydrate sources included reagent grade sucrose (Fisher Science Education, Pittsburg, PA), pectin from citrus peel (Sigma Life Science, St. Louis, MO), fructo-oligosaccharides from chicory (Sigma Life Science, St. Louis, MO), inulin from chicory (Sigma Life Science, St. Louis, MO), agave powder (Bareorganics, Scottsdale, AZ), green banana

powder (NuNaturals, Eugene, OR), black raspberry powder (BerriHealth, Corvallis, OR), baobab powder (BetterBody Foods, Lindon, UT), and pomegranate peel powder (Bixa Botanical, Maharashtra, India). Difco agar technical solidifying agent (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for preparing agar plates. Bromocresol purple (Finar Chemicals, Gujarat, India) was used a color indicator in agar plates. All media constituents were stored at 4 °C until use, except for sucrose which was stored at room temperature.

All carbohydrate sources were tested for contamination by incubating agar plates containing 2 % (wt/vol) non-autoclaved food powders in anerobic conditions for 48 hours. Because of contaminants in the purified oligosaccharide control and green banana powder (**Figure 4),** all carbohydrate sources were autoclaved with other media constituents prior to use.

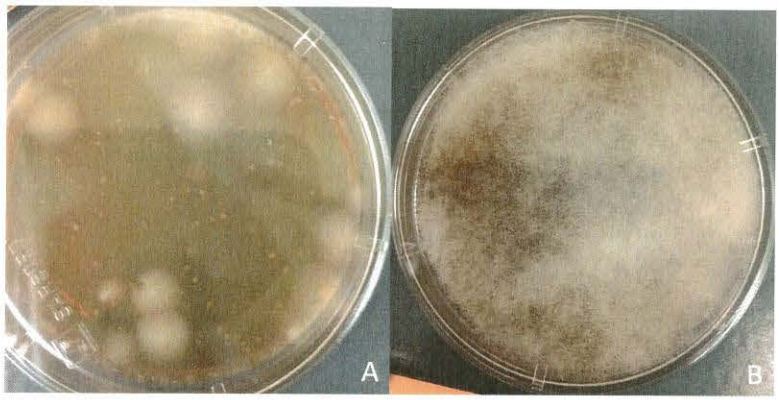

Figure 4. Contamination testing of oligosaccharide sources. Evidence of bacterial contamination of **A)** purified OS and **B)** green banana powder used for preparing culture media.

Media Preparation

Protocols for media preparation were based on a prior study assessing fermentation of FOS by probiotic bacterial strains (Kaplan and Hutkins, 2000). Base media were prepared according to package instructions by combining 3.52 % (wt/vol) MRS broth and 2 % (wt/vol)

carbohydrate source (sucrose, no carbohydrate, purified OS, agave, green banana, black raspberry, baobab, or pomegranate peel powder) in ultrapure water (Millipore Sigma, Burlington, MA). Media used for agar plates also contained 1.5 % (wt/vol) agar and 0.003 % (wt/vol) bromocresol purple indicator.

The pH of the media was checked using a standard pH meter (Thermo Scientific, Waltham, MA), and adjusted using concentrated NaOH and HCl. The initial pH of media was adjusted to the ideal range of the two bacterial strains, 5.5 – 6.2 and 6.5 – 7.0 for *L. acidophilus* and *B. lactis*, respectively (Bergey et. al, 2009 and Bergey et. al, 2012). Media were then autoclaved at 121 °C for 15 minutes. The pH of the media was then checked and adjusted for a second time. Autoclaved media were then stored at 4 °C until use.

Stock Culture Preparation

Stock cultures were prepared by adding 0.5 g of each frozen bacterial powder to 10 ml of the corresponding sucrose broth in 15 ml conical tubes. Cultures were vortexed to mix and then incubated at 37 °C for 24 hours.

Experimental Culture Preparation

Experimental broths were prepared by pouring 10 ml of broth into 15 ml conical tubes. Agar plates were prepared by melting the agar on a hot plate and pouring approximately 10 ml into 15 ml petri dishes. Dishes were then cooled in a fume hood while the agar solidified. For each bacterial strain, six broths and six plates were made for each carbohydrate source. Half of the broths and plates were used for contamination controls and half were used for bacterial inoculation.

To determine the concentration of bacteria needed to obtain ideal plate counts (30-300 colonies) a series of dilution curves was performed using sucrose broth. Initially, a 2x dilution curve was performed by diluting stock cultures down to a 1:16,384 dilution. The optical density for each dilution was determined by measuring absorbance at 620 nm using a standard spectrophotometer (Milton Roy, Houston, TX). Then, 100 µL of the 1:8 to 1:512 dilutions were plated and incubated at 37 °C for 48 hours. Plates were then observed for contamination, and colonies were counted using a darkfield colony counter. The 2x dilution curve was determined to yield plate colony counts too high, so a 10x dilution curve was performed down to a $1:1x10^8$ dilution (**Figure 5**). Additionally, due to the inability of *B. lactis* to grow in aerobic conditions (Bergey et. al, 2012), the dilution curve was repeated using an anerobic chamber and gas packs (Becton, Dickinson and Company, Franklin Lakes, NJ).

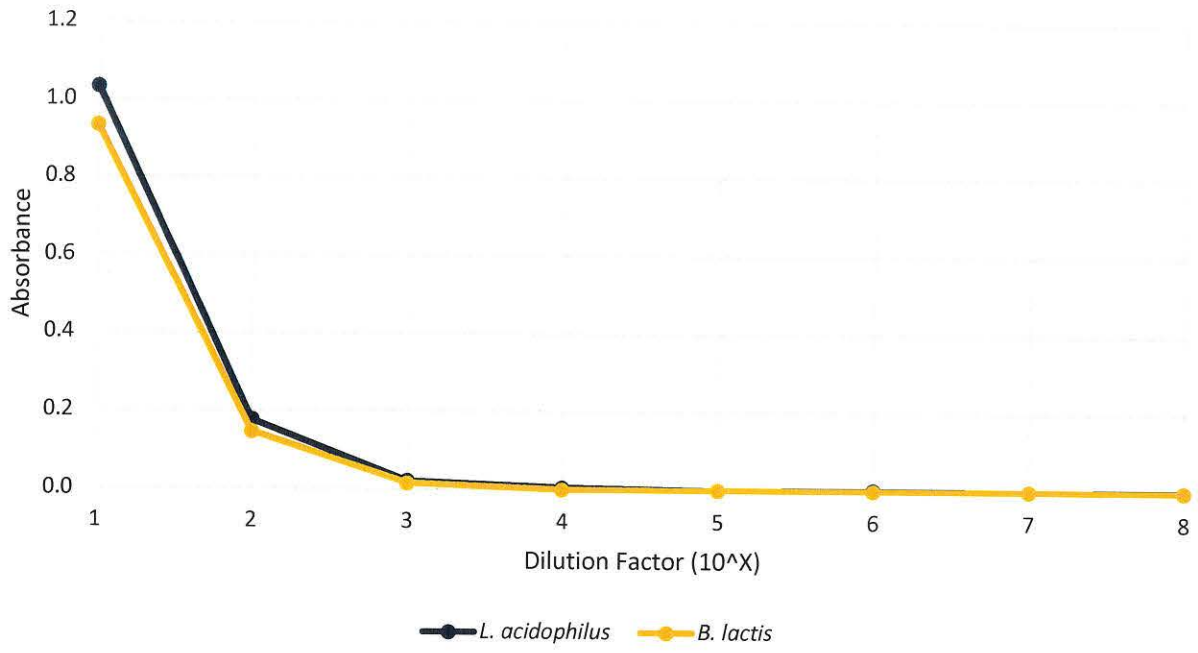

Figure 5. Initial dilution curve for both LAB strains. Absorbance measurements for *L. acidophilus* and *B. lactis* are shown with 10x dilution series ranging from a 1:10 to $1:1x10^8$.

Culture Inoculation

After establishing dilution curves for each bacterial strain, the optical densities of stock cultures where used to determine the dilution needed for inoculation of broths and agar plates. Stock cultures were diluted using broth without a carbohydrate source. *L. acidophilus* was inoculated with a $1:1x10^6$ dilution (**Figure 6**), while *B. lactis* was inoculated with a 1:100 dilution (**Figure 7**). Broths and plates were then incubated in anerobic conditions at 37 °C for 48 hours.

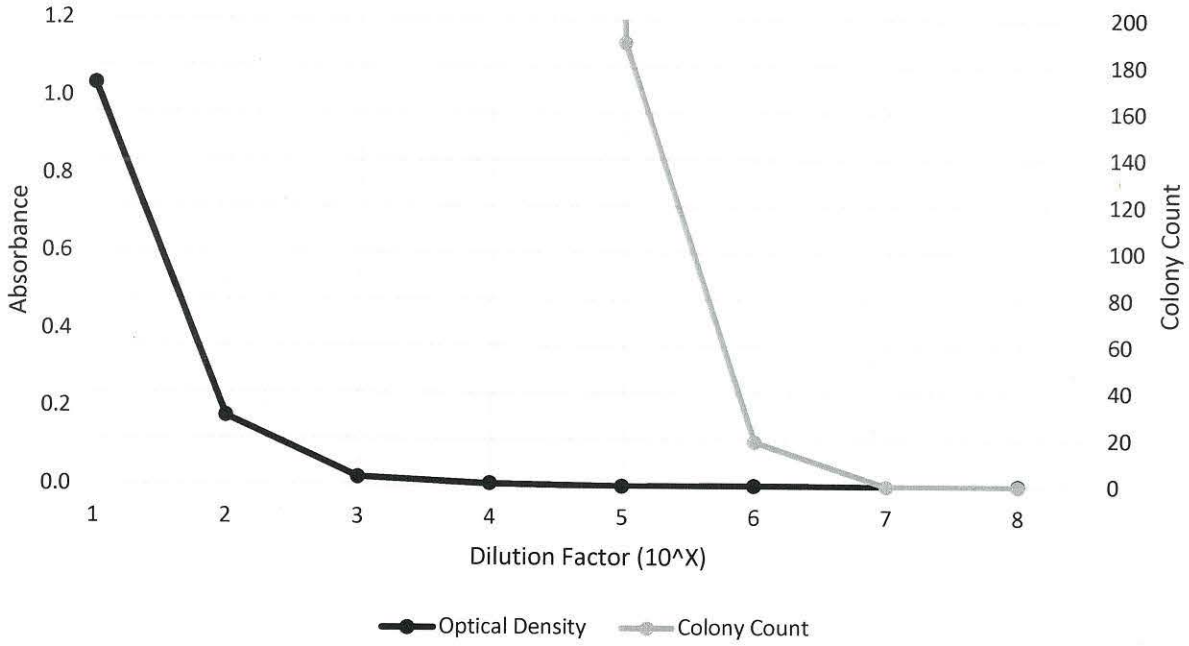

Figure 6. Dilution curve and colony counts for *L. acidophilus*. Absorbance measurements for *L. acidophilus* using a 10x dilution curve (1:10 to $1:1x10^8$) are shown along with colony counts. Dilutions from 1:10 to $1:1x10^4$ yielded colony counts >300.

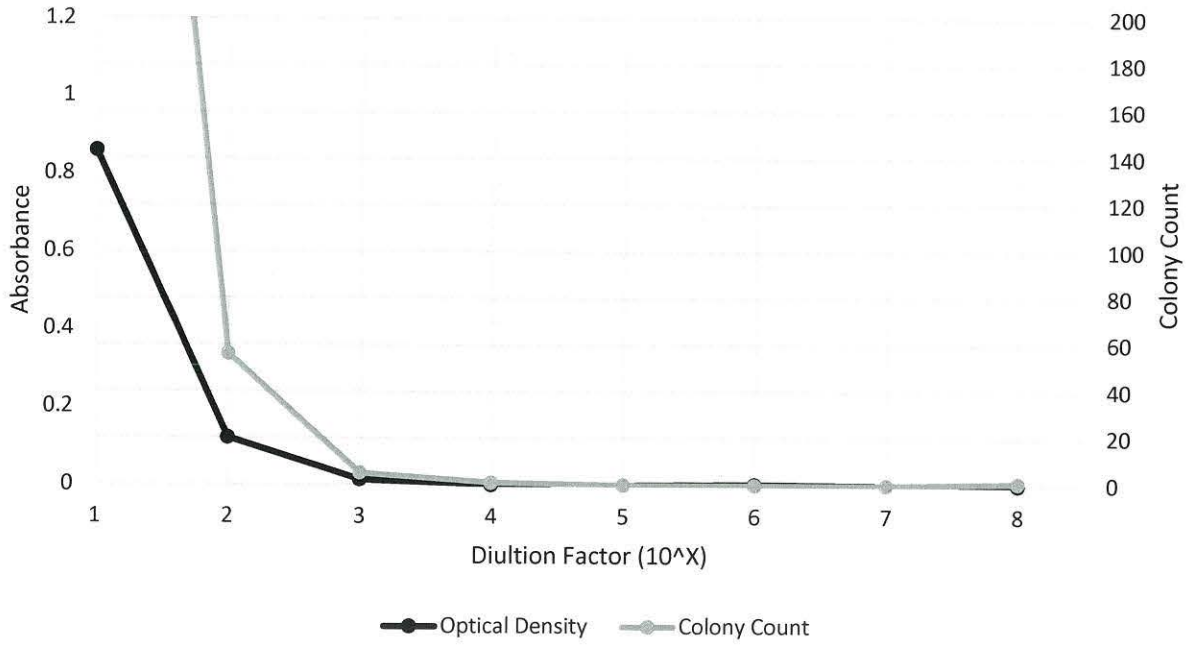

Figure 7. Dilution curve and colony counts for *B. lactis*. Absorbance measurements for *B. lactis* under anaerobic conditions using a10x dilution curve (1:10 to $1:1x10^8$ dilution) are shown along with colony counts. The 1:10 dilution had a colony count >300.

However, early experimental trials using agave powder compared to negative and positive controls suggested problems with the agar plates and the colorimetric indicator for fermentation. Under conditions of active fermentation, the bromocresol purple indicator in the agar surrounding a growing colony should turn yellow, yet this was not observed. Also, the colony counts for the *L. acidophilus* negative control were not significantly different from the positive control nor the agave powder (**Figure 8**). Thus, for this project, we elected to use only liquid broths for growing the bacteria strains in our further experimental trials. For these following experiments, broths were inoculated with 100 µL of stock culture rather than a dilution as necessary for growth on plates. The optical densities of stock cultures were recorded prior to inoculation, with *L. acidophilus* and *B. lactis* cultures having average optical densities of 1.900 and 1.805, respectively.

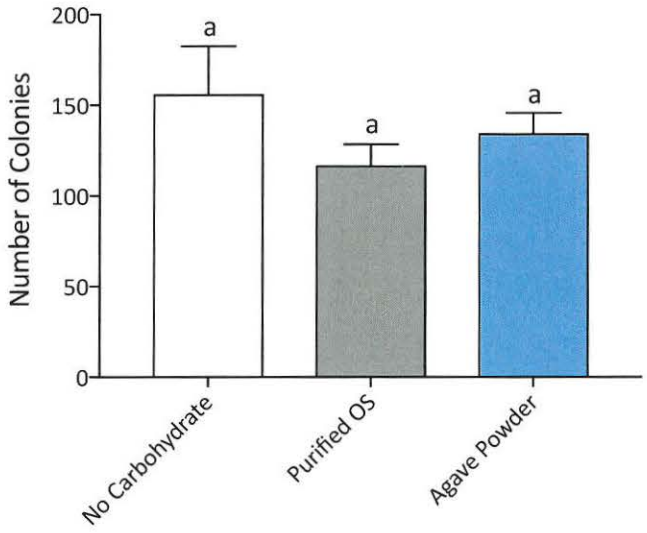

Figure 8. Initial testing for growth of *L. acidophilus* on agar culture plates. Colony counts of *L. acidophilus* on plates with no carbohydrate, purified OS, or agave powder. Different letters indicate significant difference as determined by one-way ANOVA.

Growth and Fermentation Assessment

Broths were incubated at 37 °C for 48 hours, after which the cultures were assessed for growth and fermentation. Optical density of liquid cultures was determined by absorbance at 620 nm. The original broth for each carbohydrate was used as a blank. Higher optical density is associated with a higher number of bacterial cells, indicating growth. Measuring optical density of control broths that were not inoculated with bacterial was used to test for contamination.

Fermentation of the food powders was assessed by measuring the change in media pH as compared to the original broths. Larger magnitude decreases in pH indicated greater fermentation, either a result of more efficient fermentation of the available fructooligosaccharides or as a result of bacterial growth. The calibration of the pH meter was checked after every three measurements to ensure accuracy.

Statistical Analysis

Our experimental design incorporates a 2x7 factorial design with the main factors of bacterial strain (2 levels: NCFM, HN019) and carbohydrate source (7 levels: no carbohydrate, purified OS, agave, green banana, black raspberry, baobab fruit, pomegranate peel). The experiment was repeated four times, with triplicate measurements in each trial. Technical replicates within each trial were averaged prior to statistical analysis, such that the study $n = 4$. Statistical analysis for optical density and pH change was performed using two-way ANOVA with Tukey post-hoc test for multiple comparisons (GraphPad Prism v7). Statistical analysis of colony counts was performed using one-way ANOVA with Tukey post-hoc test for multiple comparisons (GraphPad Prism v7).

RESULTS

Growth

Optical densities for both *L. acidophilus* and *B. lactis* show similar results (**Figure 9**). For *L. acidophilus*, culturing in medium with agave powder significantly increased optical density compared to both the no carbohydrate control and the purified OS ($p < 0.0001$). Green banana powder, black raspberry, or pomegranate peel on the other hand did not appear to support *L. acidophilus* growth with the medium optical density being significantly lower than the no carbohydrate control ($p < 0.0001$). Additionally, the optical density of the baobab powder was not significantly different from the no carbohydrate control and was significantly lower than the purified OS ($p < 0.0001$), suggesting that baobab does not support the growth of *L. acidophilus*. It is important to note that some of the food powders markedly altered the optical density of the

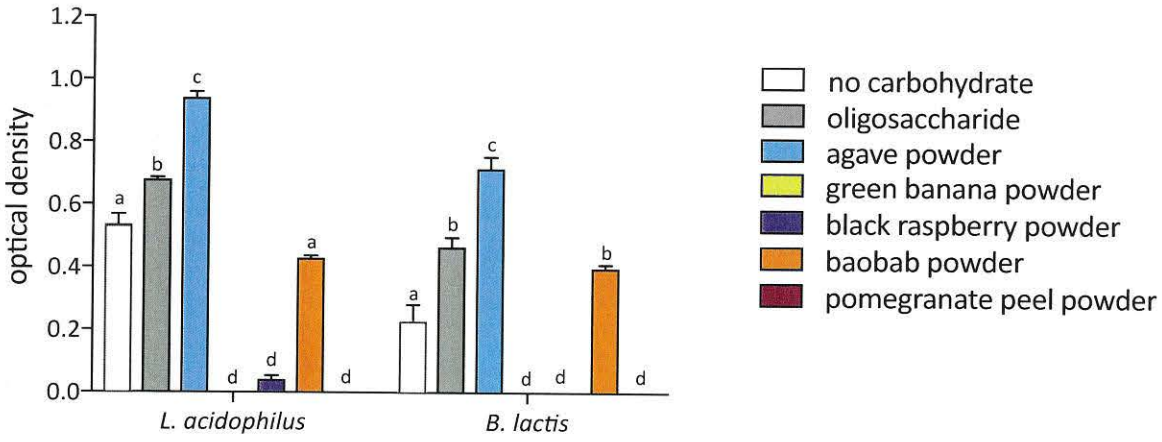

Figure 9. Assessment of bacteria growth by measurement of medium optical density change in 24 h culture. Medium optical density for *L. acidophilus* or *B. lactis* cultured in MRS broth with no carbohydrate, purified OS, agave, green banana, black raspberry, baobab, or pomegranate peel powder. Different letters indicate significant difference within bacterial strains as determined by Tukey post-hoc test following two-way ANOVA.

growth medium before culturing with bacteria, such that detection of growth of these bacteria by optical density may not be feasible. The food powders that presented this problem included the green banana, black raspberry, and pomegranate peel. Of note, mixture of agave powder with the culture media was relatively transparent, allowing for optical density measurements without difficulty. Baobab powder also seemed to produce reliable results.

For *B. lactis* (**Figure 9**), culturing in medium with agave powder significantly increased optical density compared to both the no carbohydrate control and the purified OS ($p < 0.0001$). Green banana powder, black raspberry, or pomegranate peel on the other hand did not appear to support *B. lactis* growth with the medium optical density being significantly lower than the no carbohydrate control ($p < 0.0001$). However, it is possible that these are also the result of the low transparency of these broths. Unlike in *L. acidophilus* however, the optical density of the baobab powder was not significantly different from the purified OS and was significantly higher than the no carbohydrate control ($p = 0.0005$), suggesting that baobab supports the growth of *B. lactis* as well as the positive control.

Fermentation

For both bacteria strains, there was no evidence of contamination of the control broths. In the absence of carbohydrate, the change in pH over the 24 h culture period was similar (**Figure 10**). Addition of purified OS to the *L. acidophilus* culture caused a modest, but insignificant decrease in pH, whereas the pH drop for the *B. lactis* culture was more remarkable ($p=0.0096$). However, addition of agave powder to the *L. acidophilus* culture markedly changed pH ($p<0.0001$) compared to either the negative control or the purified OS treatments. Also of note, addition of black raspberry powder increased fermentation by *L. acidophilus*, as noted by a significant drop in pH during the culture period as compared to both the negative control and

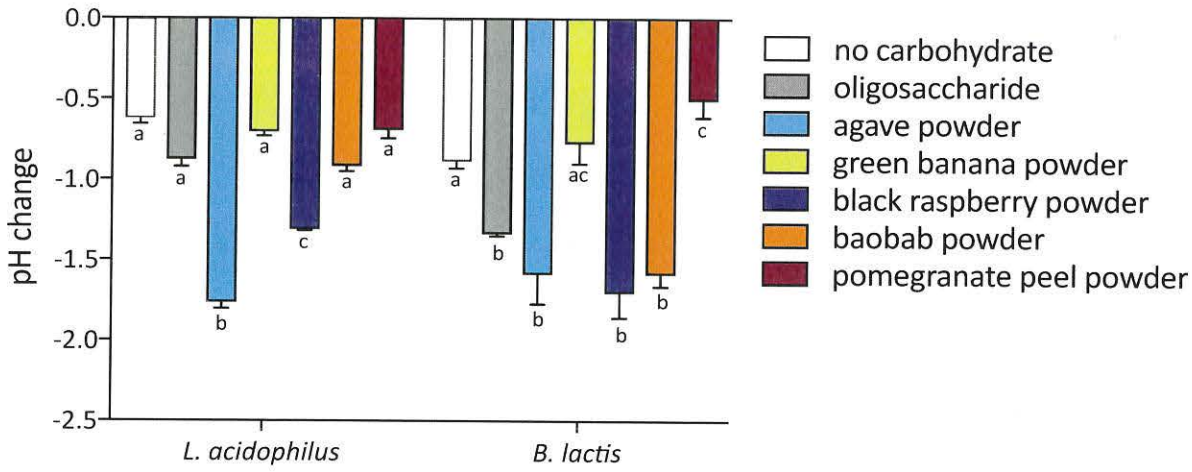

Figure 10. Assessment of fermentation by measurement of medium pH change following 24 h of culture. Medium pH change for *L. acidophilus* or *B. lactis* cultured in MRS broth with no carbohydrate, purified OS, agave, green banana, black raspberry, baobab, or pomegranate peel powder. Different letters indicate significant difference within bacterial strains as determined by Tukey post-hoc test following two-way ANOVA.

purified OS groups ($p<0.05$). Alternatively, neither the green banana, baobab or pomegranate peel powders significantly changed fermentation by *L. acidophilus* as compared to the no carbohydrate control or purified OS.

Results with the food sources of fructooligosaccharides were slightly different for the *B. lactis* culture (**Figure 10**). As noted, this strain was able to more readily ferment the purified OS. Addition of either agave, black raspberry or baobab powders also increased fermentation by B. lactis as compared to the no carbohydrate control, as indicated by significant decreases in pH ($p<0.0001$), though this fermentation was not notably different from that obtained with the purified OS. Similar to results with L. acidophilus, neither green banana or pomegranate peel powdered appeared to alter pH in cultures with B. lactis.

DISCUSSION

This project was one of the first to look at the fermentation potential of such a large variety of bioactive food powders by lactic acid bacterial strains. To our knowledge this is the first study to determine the fermentation potential of green banana, black raspberry, baobab fruit, and pomegranate peel powders by *L. acidophilus* NCFM and *B. lactis* HN019. The results from culturing both *L. acidophilus* and *B. lactis* with agave powder suggest that both bacteria strains utilize agave for growth and fermentation. These results were in line with other studies that examined the effects of agave on lactic acid bacteria, supporting the use of agave in our initial protocol design and method optimization. Based on the fermentation potential of these food powders by LAB strains, the relative OS content was determined to be highest in agave followed by black raspberry, baobab, green banana, and then pomegranate peel. In agreement with the literature, agave was found to likely have the highest OS content as it caused the greatest decrease in media pH in *L. acidophilus*, and among the highest in *B. lactis*.

Our results also suggest that black raspberry powder can be utilized by both bacterial strains for fermentation. However, it is notable that black raspberry appeared substantially more fermentable by *L. acidophilus* than *B. lactis*, as indicated by the magnitude of pH change, when compared to the purified OS. These results point to black raspberries as very promising for use as a prebiotic diet to support growth of beneficial bacteria. Such a strategy may help maintain gut microbiome homeostasis and suppress gut inflammation that often leads to tumor development.

Additionally, the fermentation potential of baobab fruit powder appeared to be dependent on bacterial strain. *L. acidophilus* did not appear to be able to utilize the baobab powder, while *B. lactis* apparently did ferment the fructooligosaccharides from baobab, as indicated by the

significant decrease in pH, on par with the purified OS. Thus, the combination of baobab powder as a prebiotic and *B. lactis* as a probiotic may offer some promise in gut inflammation and colorectal cancer dietary intervention studies.

The results from culturing both *L. acidophilus* and *B. lactis* with green banana and pomegranate powder suggests that both strains were not able to utilize these food powders for fermentation, an observation that contradicts our original hypothesis. This observation suggests that different food sources may provide different fructooligosaccharides that are differentially preferred by probiotic bacteria strains. We also note that the autoclaving step to sterilize the food powder and purified OS may have changed the chemical structures of the oligosaccharides that were ultimately provided to bacteria; thus, fermentation *in vivo* may be different than what was observed in this *in vitro* culture setting. Additionally, it is possible that some fermentation byproducts could be harmful to bacteria in culture. One study looking at the fermentation of pomegranate byproduct found growth inhibition due to decreased media quality after fermentation (Bialonska *et al.,* 2009). Thus, it is possible that green banana or pomegranate peel powders may be effectively fermented in vivo, even though we did not detect evidence for their fermentation by *L. acidophilus* or *B. lactis in vitro.*

This study had some limitations, the most significant of which was our inability to reliably estimate bacteria growth for strains grown in the food powders that markedly altered the starting optical density, such as black raspberry, green banana and pomegranate peel. An alternative measurement for growth is needed to truly evaluate the effects of these food powders, such as direct bacteria counts or the use of a different detection method that is not subject to the interference by the powder composition. Another limitation of the project was the inability to use the colorimetric indicator of fermentation for bacteria grown on agar plates. In the initial

trials, we noted that bacteria grew equally well under both negative and positive control conditions, suggesting that the agaropectin, an oligosaccharide, in the agar was sufficient for maximal bacteria growth. Additionally, varying amounts of carbohydrates in the different food powders may have affected our results, as overall carbohydrate amount was not accounted for during media preparation. While all media was prepared by adding 2 % (wt/vol) of each food powder, the total carbohydrate content added ranged from 1.12 to 1.75 % (wt/vol) and dietary fiber added ranged from 0 to 1.43 % (wt/vol). As excepted, foods containing more dietary fiber appeared to be better utilized for fermentation.

In conclusion, this study suggests that agave and black raspberry powders support lactic acid bacteria fermentation, while green banana and pomegranate peel did not under the culture conditions employed in this study. Additionally, the fermentation of baobab powder appeared to be dependent on the probiotic strain. Of the bioactive food powders tested, black raspberry appears to be the most promising candidate for future synbiotic colorectal cancer dietary intervention studies.

AUTHOR REFLECTION

As an undergraduate research fellow, I started the honors program knowing that my capstone project would be research focused, however I had no idea what type of research I would be involved with. I spent my freshman year looking for a laboratory to get involved in but was unsuccessful. It was not until my sophomore year that I found the Benninghoff laboratory and started to gain research experience. I would suggest future honors students not to worry if it takes some time to find a laboratory that is a good fit. The honors capstone is a very involved process, so it is important to find a research topic that truly peaks your interest.

My involvement with the Benninghoff laboratory began with basic animal husbandry, but quickly grew to include gross necropsy, MRI, oral glucose tolerance testing, DNA isolation, PCR, and gut microbiota analysis. Early on I had the opportunity to present my first research poster at the Student Research Symposium. These early experiences helped to shape my future work in the Benninghoff laboratory and helped to provide me with the skills and knowledge necessary to complete an independent research project.

The research process was the most difficult portion of my honors capstone. Our proposed project was outside of the regular scope of the laboratory but had the potential to play an important role in the direction of future studies. Our experience with microbiology technique was limited, and I was the only member of the team who had taken a microbiology course, though two other members were to take it in the fall. However, the Broadbent laboratory in the Nutrition, Dietetics and Food Sciences Department generously offered to provide laboratory space and expertise.

We had originally planned to finish most of the experiment over the summer and that I would then complete my portion in the beginning of the fall semester once I returned to campus. However, by the time the fall semester had started not much progress had been made. Developing and troubleshooting a protocol was much more difficult than we had imagined. This process required a lot of critical thinking and trial and error. It took most of the fall semester to finally get a working protocol and we were able to finish one experimental replicate before the semester ended. I would highly suggest students get started on their projects far in advance of their projected graduation date, as the scientific process often takes much longer than expected.

Working in an independent undergraduate research team taught me a lot about teamwork, leadership, and communication. Coordinating schedules and equally dividing the workload were some of the greatest difficulties our team faced. With my honors capstone at stake I ended up taking the lead on this project and took on a lot of the organization and planning roles for the group. At the end of the fall semester all of the other team members graduated, so seeing the project through to completion became solely my responsibility. Some of the team members planned on staying in Logan after graduation and had promised to help finish the project, but I ended up finishing the three remaining experimental replicates largely on my own. When picking a research team, I would highly recommend finding other students that are truly dedicated to the project and that will be available for its entire duration.

One of the most rewarding aspects of this process was having the opportunity to work closely with my faculty mentor Dr. Abby Benninghoff. Her continued encouragement and support have helped me to grow into the scholar and leader that I am today. I have gained so much from her knowledge and expertise, especially in science communication. Her editing style

has helped me to continue to improve upon my writing and communication skills instead of simply accepting changes. Having Dr. Benninghoff as my mentor has made the writing process of this project much more manageable than I thought it would be. Dr. Benninghoff has become someone I can turn to for help and advice, both in research and in general. Additionally, working with Dr. Benninghoff has provided me with many new opportunities, including participating in the Center for Women and Gender's Women's Leadership Initiative and being selected for the Animal, Dairy and Veterinary Sciences Department's and the College of Agriculture and Applied Sciences' Scholar of the Year. I am extremely grateful for the opportunity I have had to work with and learn from such an accomplished and knowledgeable female scientist. The best advice I can give for finding a great mentor is to find someone that you respect and look up to and who respects you in return and truly wants to see you succeed.

It has been extremely rewarding to see this project through to completion and to have the opportunity to present my findings. I love being able to share my work with others knowing that it has the potential to impact people's lives. My experience presenting at Utah Research on Capitol Hill was one of the highlights of my undergraduate career. Being able to interact with a broader audience as well as state legislators helped to expand the reach of this project and helped to show the importance of undergraduate research. I am also extremely grateful for the opportunity I had to present my research at the National Conference on Undergraduate Research. Being able to have that professional conference experience and being able to network with other undergraduate researchers from across the country and learn about their work was an amazing experience. I look forward to seeing the future impacts of my work on this field of study and potentially on human health. Knowing that my project has had a positive impact has made all of the hard work worthwhile.

Overall, undergraduate research has played an important role in defining my undergraduate career and has opened endless new opportunities. And while I do not plan on pursing a research career, the experiences I have had and the skills and knowledge I have gained will play an important role in my continued success. The ability to think critically, work in a team, and communicate effectively will be instrumental as I purse a career as a board-certified shelter medicine veterinarian. I cannot fully express my gratitude to the honors program for providing this unique experience that has helped to define my undergraduate experience and shape my future.

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Michaela Jo Brubaker was born and raised in Salt Lake City, Utah, but currently lives with her family in Medford, Oregon where she graduated from North Medford High School in 2015. Michaela returned to Utah to attend Utah State University through the Alumni Legacy Program, following in her mother's footsteps. Michaela graduated from USU in the spring of 2019 with a Bachelor of Science in Animal, Dairy and Veterinary Sciences with an emphasis in Bioveterinary Sciences and minors in Biology and Chemistry. As an honors student and undergraduate research fellow, Michaela was actively involved in research throughout her time at USU and has presented her work at a number of events including Utah Research on Capitol Hill and the National Conference on Undergraduate Research. Michaela was selected as the Animal, Dairy and Veterinary Sciences Department's and College of Agriculture and Applied Sciences' 2018-2019 Scholar of the Year. Michaela will continue her education as a member of Oregon State University's Carlson College of Veterinary Medicine Class of 2023. Michaela plans to pursue a career as a board-certified shelter medicine veterinarian.