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SYNBIOTIC ICE CREAM AS A PROBIOTIC CARRIER TESTED IN A HUMAN BLIND CROSSOVER TRIAL

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**SYNBIOTIC ICE CREAM AS A PROBIOTIC CARRIER TESTED IN A HUMAN
BLIND CROSSOVER TRIAL**

by

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A THESIS

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SYNBIOTIC ICE CREAM AS A PROBIOTIC CARRIER TESTED IN A HUMAN BLIND CROSSOVER TRIAL

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Diet is an important factor in gastrointestinal health. A synbiotic food option utilizing prebiotic and probiotic ingredients may be beneficial for improving gastrointestinal health. To conduct a human subject study of synbiotic ice cream containing prebiotic (inulin) and two strains of probiotics (*Lactobacillus casei KE99* and *Bifidobacterium bifidum*) to determine its effectiveness as a carrier for these ingredients and to identify any negative gastrointestinal side effects.

The study started with baseline data collection including a food frequency, a three day food recall, and one stool sample. The study was a 12 week crossover design with three weeks consuming placebo or treatment ice cream then a three week washout period. After, participants would switch to the other ice cream. Fecal samples were collected to examine bacteria level changes. Participants kept a log book during the two treatment periods to track gastrointestinal symptoms and record amount of ice cream consumed.

There was a not a significant difference in amount of ice cream consumed during placebo and treatment periods; $t(11) = 0.31$, $p = 0.98$. Change in reported flatulence level was not statistically significant; $t(11) = -0.82$, $p = 0.43$. There was no significant change in number of stools per day between treatment and control; $t(11) = -2.09$, $p = 0.06$. Change in Bristol scale values during placebo and treatment was not statistically

significant; $t(11) = -0.71$, $p = 0.49$. There was not significant difference in the *Bifidobacterium bifido* values during treatment ($M=47.13$, $SD=103.22$) and control ($M=24.11$, $SD=80.29$); $t(12) = 1.72$, $p = 0.11$. Synbiotic ice cream could be an effective carrier for probiotics and prebiotics. Consumption did not cause an increase in gastrointestinal symptoms. The results of the bacteria level change were not significant. Further research is needed.

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CHAPTER 1

LITERATURE REVIEW

Gastrointestinal Health

Gastrointestinal health is a very important part of overall health and can impact quality of life. In the United States colon cancer is the third most common cancer, and the risk of developing colon cancer is about one in 20, with nearly 50,000 deaths from colon cancer in the last year (1, 2). Approximately 22% of the population have chronic constipation, 5.6% have irritable bowel syndrome and over 2.2 million Americans have been diagnosed with diverticular diseases. As many as one in 10 Americans over the age of 40 have diverticulosis (3, 4). The health of the gastrointestinal tract is affected by many factors. These include pH, competition for nutrients, host conditions, metabolic interactions among bacteria and individual dietary intakes (5). Several of these factors are hard to influence with outside treatments that are non-invasive, and it is difficult to measure their effect. The main area that can be impacted with simple treatment is dietary intake. Two main categories have been reported in research literature and have measured the ability they have to improve gastrointestinal health. These are the consumption of prebiotic food ingredients, sometimes called functional foods (e.g. fructooligofructoses) and probiotic microorganisms such as *Lactobacillus* and *Bifidobacterium*).

Gastrointestinal Tract and the Gut Microbiota

The human gastrointestinal (GI) tract contains over 10^{14} microbial cells with more than 1,000 different bacterial type (6). At birth, the GI tract is sterile. The GI tract is initially colonized by facultative anaerobic bacteria. After these anaerobic bacteria remove any trace of oxygen from the environment colonizing bacteria are determined by the infant's food sources. Breastfed infants receive a wide array of microbiota from their mothers including strains of *Bifidobacterium* (7). Once at adulthood most bacteria in the

guy are non-sporing anaerobes including *Bifidobacterium* spp. and *Lactobacillus* spp. These microorganisms through fermentation break down substrates from diet such as dietary fibers and endogenous secretions. Bacteria strains have differing metabolic activities and fermentation end products which result in them being categorized as either beneficial or potentially pathogenic. The benefits from having the good bacteria strains are many. These benefits include: preventing GI tract disorders (including irritable bowel disease), preventing metabolic syndrome, improving immune response, decreasing lactose intolerance, reducing risk of getting antibiotic-associated diarrhea (especially when caused by *Clostridium difficile*), and potentially decreasing risk of colon cancer (6, 8, 9). The type and diversity of the microbiota in GI tract is also an environmental factor in obesity and the imbalance of the microbiota contribute to liver disease (8).

Probiotics

Research related to the gut microbiota has focused mainly on how it can be improved through the addition of more of the beneficial bacteria. Supplements or foods that contain these microorganisms are called probiotics. Probiotics are defined as live microorganisms that confer a health benefit on the host when administered in adequate amounts (9-11). Probiotic supplements can be found in a variety of forms including: pill, powder, capsule, gummy, and chewable. Probiotics are also found naturally in certain foods, or can be added to foods.

Taking probiotics can improve the immune response in several ways. Certain probiotics work by inhibiting adhesion and displacing pathogens for instance, *Esherichia coli*, *Staphylococcus aureus*, and *Clostridium difficile* which decreases risk of illness (12). Studies have shown that taking probiotics during antibiotic treatment can be

beneficial to maintaining healthy gut microbiome and decreasing the risk of experiencing antibiotic-associated diarrhea (13). Probiotics supplements of *Lactobacillus casei*, *Lactobacillus reuteri*, or *Bifidobacterium animalis* BB12 can be used when a patient already has acute diarrhea to decrease duration of the illness. Probiotics can be used to as treatment for someone with irritable bowel disease, ulcerative colitis, Crohn's disease and other GI disorders. Finally, probiotic usage has been linked to a decrease in serum cholesterol. Due to the benefits that probiotics have on overall health, consuming probiotics is a good choice when trying to improve overall health.

Research has been done analyzing probiotic supplements and food products for bacterial content and label accuracy. The results of these studies showed that many products have labels that are inaccurate with respect to the number of bacteria species and type of bacteria species. A few examined products did not contain the bacteria species listed, and some contained the same strain but were named differently. Some of these studies reported supplements that did not contain viable bacteria (14-18). The safety and functionality of these products is impacted by the label accuracy and as such it is important to correctly identify not only the species but the strain of bacteria used (14).

Bifidobacterium

Bifidobacterium bifidum is a bacterial species of the *bifidobacterium* genus and is one of the most common probiotic bacteria. This helpful bacteria can be found in mammals, including humans. It is a gram-positive rod shaped bacteria that is non-motile, anaerobic, and non-spore forming. It can be found living in clusters, pairs, or single units. The majority of *B.bifidum* population is found in the colon and lower small intestine, but it can also be found in breast milk and in the vagina. *B.bifidum* as part of the

gastrointestinal microflora helps the GI tract function better and reduces the chances of acute diarrhea and can help *E.coli* infections. Increasing the quantity of *B.bifidum* in the body can help boost immune function by decreasing the symptom severity and length of time a person is infected with the common cold (10). This bacteria works in the GI tract by breaking down both long and short chain simple sugars. Increasing *B.bifidum* in the GI tract can be achieved in a few ways. This bacteria can be transmitted through breast milk from the mother to the infant or it can be consumed in probiotic foods and supplements to help improve *B.bifidum* counts within the gut microbiota.

In vitro studies demonstrated that fermentation and growth rates of *bifidobacteria* increase when short chain oligofructose is the carbon source and that the chain length affects the microflora composition and activity (19-22). Numerous human studies have been conducted that demonstrate the effect of consumption of *bifidobacteria* on increasing the colonic *bifidobacteria* and subsequent return to baseline within days of discontinued consumption of *bifidobacteria* (23-25).

Lactobacillus

Lactobacillus paracasei subspecies *paracasei* is a heterofermentative lactic acid bacteria. Like *Bifidobacterium*, it is also a gram-positive rod shaped bacteria. This bacteria is commonly used in dairy product fermentation as well as probiotic supplements. Like *Bifidobacterium*, it is found in the human GI tract and found in the mouth. It is frequently used in commercial probiotic supplements or probiotic food products because it survives transit through the gastrointestinal tract well and retains functionality and viability well, especially in food products (26). *L.Paracasei* subs.

Paracasei is a beneficial bacteria that is desirable to have as part of the human gut microbiota.

Prebiotics

Numerous studies have focused on prebiotic ingredients as functional foods and how they impart a positive impact on the health of the gastrointestinal tract (9, 27-29). Prebiotics are defined as non-digestible food ingredients that positively affect the host by selectively stimulating the growth and/or activity of beneficial bacterial species (such as *Bifidobacterium* and *Lactobacillus*) in the colon, and thus improve host health (30). Non-digestible fructooligofructoses are prebiotic ingredients that have been shown to have positive effect on host health, reducing the risk of gastrointestinal diseases such as diverticulosis, diverticulitis and colon cancer (31-34). Consuming prebiotics does come with a risk of certain side effects. The side effects from consuming prebiotics can result in a higher level of flatulence and possible constipation and/or diarrhea. These side effects usually last a short period of time while the body adjusts to the ingredient. Side effects can vary depending on the type of prebiotic (27).

Fructooligofructoses are categorized by their degree of polymerization. Fructooligofructoses that have a degree of polymerization from 2-10 are named oligofructose (22, 35). Inulin is a generic term that covers all β (1 \leftarrow 2) linear molecules with a degree of polymerization (DP) varying from 2 to ~60 units (22, 25). Inulin, as a type of fructooligofructose, acts as a growth substrate for gut microflora. The bacteria that ferment the inulin gain the energy needed to grow and multiply (21, 33, 35). One study found that inulin-type fructans with a longer DP have a better prebiotic effect. This included inulin having a higher butyrate and propionate production and better stimulation

of lactic acid-producing bacteria (such as *Lactobacillus* and *Bifidobacterium*) (36). Fructooligofructoses are digested by certain types of bacteria including *Bifidobacterium* and *Lactobacillus*. When *Bifidobacteria* is the predominant bacteria in the gut, such as the case when fructooligofructoses are ingested in the adequate amounts, the number of pathogenic bacteria such as *E. coli* and *Clostridia* are decreased by competitive inhibition (22, 25, 32).

Bacteria ferment different varieties of fructooligofructoses at different speeds (21). This variation is due mainly to the difference in chain lengths as the shorter chain lengths can be broken down more quickly and the longer chains require a longer time. This variation in speed may also be correlated to location of fermentation, with longer chains being broken down in the more distal regions of the colon at a slower rate. These longer chains due to the slower rate and more distal location could lead to less side effects including less flatulence. A beneficial dose of 20g per day has been shown to be effective in producing an increase in *bifidobacteria*, although considerable individual variation existed (33).

Mixed findings have been reported for the consumption of inulin or oligofructoses (33, 37-39). Results depend on the amount and type of fructooligofructose consumed, length of time consumed, and wash out periods between treatments. Side effects (abdominal pain, distention, flatulence, constipation or diarrhea) were dependent on these same factors. Kruse, et al. concluded that long term inulin supplementation was useful and can positively change *bifidobacteria* without major gastrointestinal discomfort (39).

A project conducted by Dr. Hutkins at University of Nebraska - Lincoln focuses on assessing and enhancing stability of prebiotics in foods. In this project, the focus is on

stability of oligosaccharides in food process; specifically heat processing (baking, pasteurization and extrusion) (40).

A study was initiated by Mendlick to determine the effect of fructooligofructoses of different chain lengths on gastrointestinal parameters (41, 42). Nineteen healthy subjects aged 20-57 years old took part in a ten-week cross-over designed study. Subjects consumed either inulin or oligofructose for three weeks followed by a two-week washout period between treatments. Stool samples were collected five times (baseline, two treatments, two washout) and analyzed for *bifidobacteria*. Daily records were kept for stool frequency, stool consistency and flatulence frequency. *Bifidobacteria* counts (CFU/ml) were higher (trending toward significance) during inulin and oligofructose intakes and washout periods than baseline counts. Inulin and oligofructose treatment periods had a significant effect on stool consistency (watery/very hard) and flatulence frequency, but not stool frequency, when compared to baseline ($P < 0.05$). Further research is needed to confirm these results due to small sample size and the need for a longer washout period between treatments.

A recent study was conducted to determine what effect inulin has on pre-diabetics with regard to weight management and ectopic fat. It was an 18-week study broken into a nine week weight loss phase and nine week weight maintenance phase. Their findings showed that the inulin had two effects on diabetes risk. These effects were promoting weight loss and reducing intrahepatocellular and intramyocellular lipids in the subjects with prediabetes (43). This study illustrates that inulin could be beneficial for more than just improving gut microflora.

Synbiotics

Research has been conducted to examine what happens when probiotic bacteria and prebiotics are supplemented together. These supplements and foods are categorized as synbiotic since the probiotics and prebiotics work together synergistically to improve gastrointestinal health (9). A study of similar design to Mendlick's study was conducted to determine the microbiological effects of consuming a synbiotic containing *Bifidobacterium bifidum*, *bifidobacterium lactis*, and oligofructose in capsule form with elderly persons (41, 42, 44). The study was a double-blind randomized controlled trial with 18 participants and lasted for eight weeks consisting of three phases: a prefeeding period (1 week), the feeding period (4 weeks), and a postfeeding/washout period (3 weeks). During the feeding phase, the synbiotic group received supplements of six g of Raftilose Synergy1™ (combination of inulin and oligofructose) and a gelatin capsule containing 100 mg of a Freeze-dried probiotic containing $\sim 3.5 \times 10^{10}$ CFU each of *B. bifidum* strain BB-02 and *B. lactis* BL-01 (Rhodia). The placebo group received six g of maltooligosaccharides. All capsules were taken with a cold drink two times a day after meals. Fecal samples from weeks 1, 3, 4, 5, 6, and 8 were collected and analyzed. Throughout the feeding period both *bifidobacteria* species were detected in fecal samples from all subjects in the synbiotic group. Of these, at least one species remained detectable in fecal samples three weeks after feeding in subjects that had none of these species present during the control week. The results indicated that synbiotic consumption increased the size and diversity of protective fecal *bifidobacterial* populations, which are often reduced in older people. This study had a slightly longer washout period than the previous, and had positive results.

A study using yogurt by Palaria, et al. had a similar design. It was divided into five consecutive periods: a pre-feeding period (1 week), a feeding period (3 weeks), a washout period (4 weeks), a second feeding period (3 weeks), and a final washout period (4 weeks) (45). Fecal samples were collected at the start and at the end of the first week. During the first feeding period, the subjects daily consumed either 94 g of placebo, which consisted of milk acidified to pH 4.2 with lactic acid, or 94 g of a drinkable yogurt containing 10^9 to 10^{10} CFU of strain *B. animalis* subsp. *lactis* Bb-12 and 1 g of inulin per serving. The yogurt was prepared with skim milk and a standard yogurt starter blend consisting of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, together with the *B. animalis* subsp. *lactis* Bb-12 culture and had a final pH of 4.2. Fecal samples were collected at the end of each of the three (3) weeks. The subjects consumed neither the yogurt nor the placebo during the subsequent washout period. Single fecal samples were collected at the end of every two (2) weeks. A live/dead PCR procedure indicated that the Bb-12 microorganism was detected in the fecal samples was alive. A significant increase ($P < 0.001$) in the total *bifidobacterial* numbers was observed in both groups of subjects during the final washout period compared to the prefeeding period. This increase in total *bifidobacteria* corresponded with a significant decrease ($P < 0.05$) in numbers of *clostridia* but not *enterobacteria*.

Prebiotic, Probiotic and Synbiotic Ice Cream

Many studies have been conducted to determine if ice cream would be an effective carrier for prebiotics, probiotics, or synbiotics. These studies were conducted mostly to determine the palatability or sensory acceptability of these products as well as their ability to keep the bacteria viable (41, 46).

The Wood and Lum and Albrecht project objective was to determine consumer acceptability of ice cream with prebiotic ingredients. A commercial ice cream mix was made substituting 0%, 10%, 20%, or 30% of the sugar for either Fructooligosaccharides (FOS) or inulin. Participants rated the synbiotic ice cream for its sensory attributes of sweetness, smoothness, and vanilla flavor (47, 48). When 10% and 20% inulin ice cream were compared to the control (0%), no significant differences in sweetness, smoothness, vanilla flavor or overall acceptability were found ($P < 0.05$). The 30% inulin ice cream was significantly less sweet than the control and 10% and 20% inulin ice cream. The 30% inulin was less smooth and had less vanilla flavor than the control, and was less acceptable than both the control and the 10% inulin ice cream ($P < 0.05$). For 10% and 20% FOS ice cream, no significant differences were found in sweetness, smoothness, vanilla flavor or overall acceptability compared to the control ($P < 0.05$). These results suggest that FOS and inulin may be acceptable ingredients in ice cream when substituted up to 20% of the sugar.

A few studies examined the use of just probiotics in ice cream to determine if it would be a viable probiotic carrier. The strains used in the first study included: *Lactobacillus acidophilus*, *Bifidobacterium bifidum*. Sensory results were positive but the bacteria counts decreased throughout the 90-day storage, however did maintain their probiotic qualities (49). The second study illustrated that ice cream is not good at maintaining the viability of the bacteria. However, if the ice cream is made with a prebiotic such as FOS or inulin, these ingredients help to maintain the viability of the bacteria (50). In both studies, the ice cream was made by inoculating some of the milk and then adding it after cooling down the rest of the ingredients. Cruz and colleagues

reviewed the potential for ice cream to be a probiotic food carrier. Two studies with probiotics in ice cream reported that the bacteria survived the freezing process (51). Only one study could be found where they actually tested a probiotic ice cream formula in a human trial to determine its effects. This study examined the impact of probiotic ice cream consumption on levels of *Salivary Mutans Streptococci* (SMS) during and after the trial. They found that the levels of the SMS decreased during treatment, but by six months post treatment, the SMS levels were similar to baseline (52).

Several studies could be found which examined the effectiveness of prebiotics and probiotics used together synbiotically. One study examined the differences between prebiotic, probiotic and synbiotic ice cream. Their probiotic ice cream contained two *Lactobacillus* species (*L. rhamnosus* and *L. casei*). These were used independently within fruit or vanilla flavored ice cream. Inulin (2.5%, 5% or 10%) replaced part of the stabilizer for the prebiotic ice cream and the synbiotic ice cream used either 3 or 6% inulin with either of the probiotic organisms. All ice cream samples were effective at maintaining probiotic function (46).

Two UCARE students (Lim and Mills) examined the sensory characteristics of a synbiotic ice cream in which 10 %, 20% or 30% of the sugar was substituted with either inulin or fructooligosaccharide (FOS) (53). The probiotic species, *Lactobacillus casei* KE99 (0.3g) and *Bifidobacterium bifidum* (0.3g), were formulated in the ice cream products to make a synbiotic ice cream. Both probiotics used were purchased as freeze dried cultures from ProbioFerm in Des Moines, Iowa (54). These probiotics were labeled as 100% pure and food grade. Their results reported that the 30% FOS negatively affected the flavor and texture. The addition of inulin did not affect the sweetness,

smoothness, flavor, texture, and overall acceptability of the ice cream treatments. The addition of the probiotics did not affect any of the sensory characteristics of the ice cream samples. Another UCARE student (Irby) then examined the viability of the probiotics during ice cream storage (55). The results of this study demonstrated that the probiotics, *Lactobacillus casei* KE99 (0.3 grams) and *Bifidobacterium bifidum* (0.3 grams) continued to be viable over a four month period (120 days), making these bacteria a good choice to use as a probiotic and the ice cream a good synbiotic carrier. These two strains were the same strains chosen for our research study.

Recent studies examined the effectiveness of using microencapsulation (MEP) to determine the effect on bacteria survival within synbiotic ice cream. The results of all the studies have reported slower reduction in probiotic bacteria over storage time (56-59). The best results were reported when the MEP bacteria was incorporated into chocolate particles (57). Overall, all showed MEP to be an effective method for maintaining probiotic viability within ice cream. These studies were all published after our study design was set and underway.

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CHAPTER 2

Problem Statement

Based on the literature review and previous research conducted, the objectives of our study are to determine if there are any significant gastrointestinal side effects from consuming synbiotic ice cream as well as to examine how the bacteria counts change prior to eating the ice cream, during consumption, and post consumption.

CHAPTER 3

Article

Synbiotic Ice Cream as a Carrier for Prebiotic Inulin and Probiotics *Lactobacillus casei KE99* and *Bifidobacterium bifidum* Tested in a Human Crossover Trial

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ABSTRACT

Background: Diet is an important factor in gastrointestinal health. A synbiotic food option utilizing prebiotic and probiotic ingredients may be beneficial for improving gastrointestinal health.

Objective: To conduct a human subject study of synbiotic ice cream containing prebiotic (inulin) and two strains of probiotics (*Lactobacillus casei* KE99 and *Bifidobacterium bifidum*) to determine its effectiveness as a carrier for these ingredients and to identify any negative gastrointestinal side effects.

Methods: The study started with baseline data collection including a food frequency, a three day food recall, and one stool sample. The study was a 12 week crossover design with three weeks consuming placebo or treatment ice cream then a three week washout period. After, participants would switch to the other ice cream. Fecal samples were collected to examine bacteria level changes. Participants kept a log book during the two treatment periods to track gastrointestinal symptoms and record amount of ice cream consumed.

Results: There was not a significant difference in amount of ice cream consumed during placebo and treatment periods; $t(11) = 0.31$, $p = 0.98$. Change in reported flatulence level was not statistically significant; $t(11) = -0.82$, $p = 0.43$. There was no significant change in number of stools per day between treatment and control; $t(11) = -2.09$, $p = 0.06$. Change in Bristol scale values during placebo and treatment was not statistically significant; $t(11) = -0.71$, $p = 0.49$. There was not significant difference in the *Bifidobacterium bifido* values during treatment ($M=47.13$, $SD=103.22$) and control ($M=24.11$, $SD=80.29$); $t(12) = 1.72$, $p = 0.11$.

Conclusion: Synbiotic ice cream could be an effective carrier for probiotics and prebiotics. Consumption did not cause an increase in gastrointestinal symptoms. The results of the bacteria level change were not significant. Further research is needed.

Key words: probiotic, synbiotic, prebiotic, synbiotic ice cream, *Bifidobacterium bifidum*, *Lactobacillus casei KE99*

INTRODUCTION

Human gastrointestinal health may be improved by the consumption of prebiotic food ingredients and foods containing probiotic microorganisms such *Lactobacillus* and *Bifidobacterium* (1).

Prebiotics are defined as “nondigestible food ingredients that have a beneficial impact on the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improving host health” (2, 3). These prebiotics are nondigestible because the host, humans, lack the enzymes required to break the beta (β) bonds that hold the prebiotic together. These prebiotics reach the colon still intact since the host cannot break them down. Certain strains of beneficial bacteria found in the gastrointestinal tract, especially *Bifidobacterium* and *Lactobacillus*, are able to break the β bonds in the prebiotics providing an energy source for the bacteria and as well creating byproducts of fermentation that are beneficial to the host.

Nondigestible fructooligofructoses, including inulin, are prebiotic ingredients that have been shown to have positive effects on host health, reducing the risk of gastrointestinal diseases such as diverticulosis, diverticulitis, and colon cancer (4-9). Inulin is a generic term that covers all β (1 \leftarrow 2) linear molecules and their degree of polymerization (DP) varies from 2 to ~60 units (10, 11). The inulin then serves as a growth substrate for the gut microflora (8, 12, 13).

Mixed findings have been reported for the consumption of inulin or oligofructoses (8, 14-16). Results depend on the amount and type of fructooligofructose, consumed, length of time consumed, and wash out periods between treatments. Some subjects have experienced side effects from consuming inulin that range from an increased level of

flatulence to possible constipation and/or diarrhea (4). Findings show that bacteria ferment different chain lengths at different speeds. The differing speeds may be correlated to location of fermentation, with the longer chains being broken down in the more distal regions at a slower rate. The longer chains at a slower rate could lead to less side effects, including less flatulence (17). A beneficial dose of 20g per day has been shown to be effective in producing an increase in *bifidobacteria*, although considerable individual variation existed (8). When *Bifidobacteria* is more predominant in the gut, such as the case when inulin and other fructooligosaccharides are ingested in the proper amounts, the number of pathogenic bacteria such as *E. coli* and *Clostridia* are decreased by competitive inhibition which can improve GI health (7, 12, 13).

Numerous human studies have been conducted that demonstrate the consumption of *bifidobacteria* increases colonic *bifidobacteria*, and when stopped, levels return to baseline (10, 18, 19). In vitro studies demonstrated that fermentation and growth rates of *bifidobacteria* increase when short chain oligosaccharide is the carbon source and that the chain length affects the microflora composition and activity (11, 14, 20, 21). According to a Health and Human Services report, previous research with probiotics has been very inconsistent when describing the intervention, reporting the results of the intervention, and providing information regarding genus, species, and strain. Improving these aspects in future research was recommended (22). The safety and functionality of these products is dependent on labeling accuracy. Unfortunately accurate labelling has not always been found (23-25). Rijkers and colleagues noted that adverse events were not well described and the food matrix used for the probiotic was not identified (26).

Recently, many studies have investigated the co-administration of prebiotics and probiotics called synbiotics. Synbiotics may help enhance the benefits provided by either pre- or probiotic supplementation (3, 27, 28). Studies have been conducted to evaluate the sensory properties of either probiotic, prebiotic, and synbiotic ice creams (29-31). For their probiotic ice cream, two *Lactobacillus* species (*L. rhamnosus* and *L. casei*) were used independently with either fruit or vanilla flavors. Inulin (2.5%, 5% or 10%) replaced part of the stabilizer for the prebiotic ice cream and the synbiotic ice cream used either 3 or 6% inulin with either of the probiotic organisms (29). Another study tested the palatability of prebiotics replacing 10%, 20%, and 30% of the sweetener with inulin or FOS. The 10% was found to be the most tolerated with the 20% being acceptable and the 30% not accepted. This same prebiotic ice cream was then tested as a synbiotic mixture with probiotic species, *Lactobacillus casei* KE99 (0.3g) and *Bifidobacterium bifidum* (0.3g) added and the same tolerance levels were found (31). Cruz and colleagues reviewed the potential for ice cream to be a probiotic food carrier. Two studies with probiotics in ice cream showed they survived the freezing process (30). Clinical studies have not been conducted with the probiotic, prebiotic or synbiotic ice cream formulations.

The objective to our study was to determine if there are any significant gastrointestinal side effects from consuming synbiotic ice cream as well as to examine how the bacteria counts change prior to eating the ice cream, during consumption, and post consumption. For this study a randomized, blind, crossover, placebo controlled human trial of synbiotic ice cream containing the prebiotic inulin and probiotics *Lactobacillus casei* KE99 and *Bifidobacterium bifidum* was performed to test the

effectiveness of ice cream as a carrier for prebiotic and probiotic ingredients and examined the impact on gastrointestinal health.

SUBJECTS AND METHODS

Study Design

The study was a 13 week blind, placebo-controlled crossover trial where participants were randomly assigned and either consumed synbiotic ice cream or plain (placebo) ice cream for a three-week treatment period followed by three-week washout period as suggested by the design of other studies and the findings on these particular bacteria strains (32-34). The three-weeks of treatment followed by three-weeks of washout was then repeated with the opposite ice cream type, either synbiotic or placebo.

Baseline data was collected the week prior to the start of the first treatment of the study. At the start of each week of the three week treatment periods, participants received seven one-half cup containers of ice cream, to be consumed daily for the next week. At the end of that week, participants brought in their stool sample and received next week's supply of ice cream. In blinded fashion, these portion cups for the treatment and placebo were identically packaged

Ice Cream Formulation

The ice cream formulation used was the same formulation that had been tested previously for sensory qualities and probiotic stability. The bacteria were pure freeze-dried food grade probiotics (*Lactobacillus casei* KE99 and *Bifidobacterium bifidum*) purchased from ProbioFerm in Des Moines, IA. In each serving of synbiotic ice cream participants were consuming approximately 0.7 g of inulin and 0.017 grams or 1 billion (1×10^9) CFU per serving of the probiotic bacteria. All ice cream for the synbiotic

treatment as well as the control was made prior to starting a treatment period. The synbiotic ice cream was made using the following formulation (Appendix 1):

Ingredients	Control	Synbiotic W/10% inulin
amounts in grams		
Milk	595	595
Cream	202	202
Nonfat Dry Milk	50	50
Corn Syrup Solids	45	45
Stabilizer	7	7
Sugar	100	90
Inulin	0	10
<i>B.bifidum</i>	0	0.3
<i>Lacto casei</i>	0	0.3

To prepare the ice cream: Dry ingredients were mixed in with the wet ingredients and the ice cream preparation was heated in a microwave during two separate intervals. Inulin is obtained in powder form from the manufacturer SourceNaturals. After four minutes, the ice cream preparation was stirred and then put back in the microwave for three and a half minutes to solubilize the stabilizer. After heating, the ice cream batch are cooled using an ice bath until they reached a temperature below 40°C so that the probiotics could be at a temperature in which their viability would not be compromised. *Bifidobacterium bifidum* and *Lactobacillus casei* KE99 were obtained from ProBioFerm (Des Moines, IA) in 50 billion colony forming units (CFU) per gram. After adding the probiotics the batch can be poured into an ice cream maker and removed once the ice cream is done churning. Note: These steps are followed for both recipes minus the addition of the probiotics and prebiotics to the control. Once the machine finished the ice cream was measured out into half cup servings and stored in individual plastic containers

with lids. These were then stored in a standard freezer until they were given to the participants.

Participants

The study was approved by the Institutional Review Board and all volunteers gave written informed consent prior to the start of the project. Participants were recruited through posters placed in campus buildings (Appendix 2). Emails were sent out to the current and previous class of dietetic interns upon approval of the internship program director (Appendix 3). Subjects had to be a student or faculty member of University of Nebraska – Lincoln for ease of safety with regard to any potential health concerns. The participants filled out a screening tool to determine if they met the requirements to be participants (Appendix 4). Potential subjects with a history of colon diseases, diverticulosis or diverticulitis, chronic diarrhea or constipation, and recent antibiotics in the last six weeks were excluded from the study, as were women who were pregnant or breastfeeding. Participants were asked to read through and sign a document of informed consent (Appendix 5). All participants were asked to continue following their normal diet routine and not modify it in any way. In particular, they were asked not to increase probiotic or prebiotic consumption from their usual intake.

Sampling

A food frequency, a three-day diet recall, and a stool sample were collected at baseline (Appendix 6). Then participants filled out a daily log book during both treatments periods to track stool/flatulence frequency, stool consistency, and percent of ice cream consumed as well as providing them with space to log any additional comments (Appendix 7). The participants were asked to provide a total of 19 stool

samples throughout the study; one at the end of each week, plus one for each of the first three days of the washout periods. These were to be collected on days 0, 7, 14, 21, 22, 23, 24, 28, 35, 42, 49, 56, 63, 64, 65, 66, 70, 77, 84 (Appendix 8). Samples were brought to the researcher utilizing the collection tubs that the participants used to collect the samples. These samples were held at -20°C until processing.

Food recall and Food Frequency

Food recalls were collected and analyzed for each participant to determine a baseline of how much fiber they usually consume as well as how many probiotic food items they usually consume. The food recalls were analyzed using USDA's SuperTracker to get a consistent and accurate picture of the participants' usual intake (35) (example pages Appendix 9-10). The food frequency was used as a supplemental piece to view how much the participants reported that they usually consume of various prebiotic and probiotic food sources. The food frequency tool was designed specifically for this study but utilized information on high and moderate prebiotic and probiotic food sources.

Log book

Participants were given log books to keep track of the following during the two treatment periods: ice cream consumed (percentage scale from 0% to 100%), flatulence level (Likert scale from 0 to 10), number of stools, and the Bristol scale for stool consistency.

Preparation of cultures

In preparation for analysis of the fecal samples procedures had to be completed on the same pure freeze dried cultures from ProbioFerm (Des Moines, IA) used within the ice cream. The cultures were grown in Difco Lactobacilli MRS Agar and Difco

Lactobacilli MRS broth (Becton, Dickinson and Co., Franklin Lakes, NJ) and incubated in an airtight container with an GasPak™ EZ Anaerobe Container System with Indicator (Becton, Dickinson and Co., Sparks, MD) at 37°C overnight. Growth was checked the following day. If there was not enough growth they were left in overnight to continue growing. If growth was seen the sample was removed from the incubator and the next steps were taken. Growth on the plate was examined. One or two well isolated colonies would be picked with a sterile loop and streaked onto a new MRS agar plate. The incubation step was repeated as above followed by streaking of one or two well isolated colonies from each plate. This was done a minimum of four times to ensure purity of the culture.

An isolated colony was picked from each plate and stained using a gram stain kit. Both *B.bifidum* and *L.casei* are gram positive rods found in clusters, pairs, or independently. The gram stained cultures were examined under a microscope to determine if the cultures were the correct stain color, correct shape, and colony formation style. One or two times the slides showed a mix of cocci and rods. The steps of isolation, growth, and staining were repeated until specimens under the microscope looked correct. Once the gram stain results showed the correct shape, colony formation, and gram stain results, isolated colonies were picked with a sterilized loop and placed in Difco Lactobacilli MRS broth (Becton, Dickinson and Co., Franklin Lakes, NJ) for a final incubation with the same condition as previously described. The next day samples were taken out and vortexed briefly to mix. 1 mL of sample was extracted and centrifuged at 1,700xg for three minutes, supernatant was discarded.

DNA was extracted using QuickExtract™ Bacterial DNA Extraction Kit (Epicentre, Madison, WI) with the following changes to the protocol: The sample pellet from above was used and 500 μ L quick extract was added to the tube containing the pellet. Sample was Vortexed for 45 seconds, incubated at 65°C for ten minutes, vortexed for 30 seconds, then incubated at 95°C for two minutes.

The bacterial DNA was then amplified using primers 27F and 1492R (barcoded) (36). The PCR reactions were performed in 20 μ L volumes and contained 0.5 μ L of Terra DNA polymerase (Clontech Laboratories, Mountain view, CA), 10 μ L reaction buffer, 250 nL of each primer, and 1 μ L of the extracted nucleic acid template or no-template control. The cycling conditions were an initial denaturation of 98°C for 2 minutes, followed by 35 cycles of 98°C for 30 seconds, 52°C for 30 seconds, and 68°C for 90 seconds; and a final extension of 68°C for 4 minutes. Following amplification, PCR products were analyzed on a 1.5% agarose gel to confirm correct product size.

Once completed the concentration level of each sample was checked. This was done using the NanoDrop® ND-1000 Spectrophotometer. Cultures were then diluted with PCR grade water to get all samples to desired level of 10-15 ng/ μ L. 10 μ L of sample was combined with 2 μ L of 518R primer and sent to Eurofins genomics (Eurofins MWG Operon LLC., Louisville, KY) for sequencing. Despite the fact that the cultures were isolated from samples grown from the pure freeze dried cultures the sequencing results either had too many unidentified bases (N) in sequencing to have a clear result or they matched better to a different bacteria. Both of these results meant that the sequence was not strong enough, either due to lack of base pair information or impurity, to be used to

make probes. As a result it was determined that primers for the two bacteria should be used.

Primer Selection

Several sets of primers were designed using the primer designed software at IDT (<http://www.idtdna.com/primerquest/home/index>) using default parameters, for the bacterial strains to determine the one that worked best with our specific cultures. To select the best set of primers, the primer pairs were tested against four pure cultures of each *B.bifidum* and *L.casei* by using PCR and gel electrophoresis to determine which would amplify the desired cultures and not the others (36). The PCR reactions were performed in 20 μ L volumes and contained 0.5 μ L of Terra DNA polymerase (Clontech Laboratories, Mountain view, CA), 10 μ L reaction buffer, 250 nL of each primer, and 1 μ L of the extracted nucleic acid template or no-template control. The cycling conditions were an initial denaturation of 98°C for 2 minutes, followed by 35 cycles of 98°C for 10 seconds, 52°C for 30 seconds, and 68°C for 60 seconds; and a final extension of 68°C for 4 minutes. Following amplification, PCR products were analyzed on a 1.5% agarose gel to determine what the primers had amplified. Initially, three sets of primers for both *B.bifidum* and *L.casei* were purchased. The first attempt with all primers and all eight samples resulted in one promising primer set for each which showed amplification of the matching four samples and no amplification of the other four. These two were run through PCR again with the same volume and mixture. The cycling conditions were modified so that the middle part of the cycle (previously 52°C for 30 seconds) had a gradient temperature of 54°C, 56°C, 58°C, 60°C, 62°C. The results were examined on a 1.5% agarose gel. The results were that the *B.bifidum* 2 primer set was selected and

determined to work best at 60°C annealing temperature (see Appendix 11 for full sequence information and Figure 1 for gel image). Unfortunately, the gel results showed that the *L.casei* primer had amplified all eight samples and not just the four *L.casei* samples illustrating that it was not selective enough to be used (see Figure 1).

The selected *B.bifidum* primer and all three *L.casei* primers were tested again using SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) this time since this would be the mix used with the participant samples for quantification of the bacteria. The PCR reactions were performed in 15 µL volumes and contained 7.5 µL SYBR® Green PCR Master Mix, 250 nL of each primer and 4µl of the extracted nucleic acid template. The cycling conditions were an initial denaturation of 95°C for 2 minutes, followed by 35 cycles of 95°C for 10 seconds, gradient of 54°C and 60°C for 30 seconds, and 68 °C for 60 seconds; and a final extension of 68 °C for 5 minutes. This gave the same results as the before with *B.bifidum* primer 2 being selected *L.casei* primers not selective enough. Six new sets of *L.casei* primers were purchased for testing. These were tested with all four *L.casei* pure cultures and one *B.bifidum* culture and the same SYBR® Green PCR in volume and ratios listed above. The cycling conditions were modified to include a wider temperature gradient with a gradient temperature set of 54°C, 56°C, 58°C, 60°C, 62°C. The results from testing with these new *L.casei* primers still resulted in no *L.casei* primer being selected due to either lack of selectivity and incorrect selectivity, meaning it did not amplify this exact strain (see Figure 2). Due to time constraints with this project the next steps were completed using just the primers selected for *B.bifidum*.

Fecal samples analysis

With the participant samples, the PowerMag™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) was used to extract the DNA from the stool samples following the manufacturer's protocol with the following modifications: the 2 bead-beating steps were performed in a TissueLyser (QIAGEN Inc., Valencia, CA) and samples were incubated in a 95 °C water bath for 5 min between bead-beating steps.

After DNA was extracted from the samples, their concentration levels were tested using the NanoDrop® ND-1000 Spectrophotometer. Once the concentration was identified the epMotion M5073 liquid handler (Eppendorf AG, Hamburg, Germany) was used to normalize all samples to 10ng/μL. This way all concentration levels were equivalent before quantifying the bacteria enabling us to get a more accurate picture of the level changes throughout treatment.

The samples were then processed through quantification Polymerase Chain Reaction (qPCR) using the 7900HT Fast Real-Time PCR System in duplicate. All these were done using the same SYBR® Green PCR Master Mix volumes and ratios mentioned above. The V3 region of the 16S rRNA gene specific to eubacterial communities was amplified using universal primers 341F and 518R (barcoded). The cycling conditions were an initial denaturation of 98°C for 10 minutes, followed by 30 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds; and a dissociation step of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. Next the samples were processed through qPCR with the selected *B.bifidum* primers. The cycling conditions were an initial denaturation of 98°C for 2 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 68°C for 45 seconds; and a dissociation step of 95°C

for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. The dissociation curve for each of the reactions was examined upon completion and samples with a substandard curve were re-done (example of one of the Dissociation curves found Appendix 12). The quantified results of the sample sets for the 16s and the *Bifidobacteria* were averaged for each participant. These were then quantified using Δct , $\Delta\Delta\text{ct}$ and fold change ($2^{(-\Delta\Delta\text{ct})}$). The data from these experiments was analyzed using SPSS using a two tailed t-test to compare the changes in *Bifidobacteria* count between the treatment and control times of the study.

Objective

The objective of this study was to conduct a clinical feeding study to determine if synbiotic ice cream is a viable food carrier for prebiotic (inulin) and probiotic (*Lactobacillus casei* KE99 and *Bifidobacterium bifidum*) food ingredients and to determine if it had any negative gastrointestinal effects on the participants.

RESULTS and DISCUSSION

Results

Demographic

Initially, 15 participants started the study. The study ended with 13 participants; two males and 11 females or 13% males and 87% females (Figure 3) Participants were between 21 and 28 years (Figure 4) The average age was 23 years.

Food Recall and Food Frequency

Prior to the start of the study, participants filled out a food frequency and a food recall. The food frequency showed participants consumed an average of 3 probiotic foods

per week and 36 prebiotic food per week (Figure 5). The food recall results showed an average of 19.5 grams of fiber per day and 0.3 probiotics a day (Figures 6 & 7).

Log Book

Results of the participants log book (Appendix 7) are reported in the table 5.

There was a not a significant difference in the scores for the percentage (from 0%-100%) of amount of ice cream consumed during the placebo period (M=92.76, SD=9.74) and the amount of ice cream consumed during the treatment period (M=92.67, SD=9.27); $t(11)=0.31$, $p = 0.98$. There was not a significant difference in the reported flatulence level (likert scale from 0-10) during placebo (M=3.74, SD=1.56) and during treatment (M=4.02, SD=1.61) periods; $t(11)= -0.82$, $p = 0.43$. There was a not a significant difference in the reported number of stools per day during placebo (M=1.43, SD=0.51) and during treatment (M=1.69, SD=0.74) periods; $t(11)= -2.09$, $p = 0.06$. There was not a significant difference in the reported number of stools per day during placebo (M=1.43, SD=0.51) and during treatment (M=1.69, SD=0.74) periods; $t(11)= -2.09$, $p = 0.06$. There was not significant difference in the reported Bristol scale values per day during placebo (M=3.18, SD=0.17) and during treatment (M=3.35, SD=0.82) periods; $t(11)= -0.71$, $p = 0.49$.

Bacterial

There was not a significant difference in the *Bifidobacterium bifido* fold change values over the treatment (M=47.13, SD=103.22) and over the control (M=24.11, SD=80.29) periods; $t(12)= 1.72$, $p = 0.11$ (Table 3.6).

Discussion

After collecting and analyzing all of the samples and materials the treatments shows no statistically significant impact on gastrointestinal symptoms of the participants. The lab analysis results show no statistically significant change as to effectiveness and impact on colonization and viability in the gastrointestinal tract. This lack of statistical significance was mostly due to the small sample size.

Participation

Two participants did not complete the study. One dropped out in two weeks after being placed on antibiotic treatment, thus disqualifying them from continuing with the study. Another participant dropped prior to the midpoint of the study due to time constraints. No participants discontinued participation due to any complications caused by treatment from the study. The remaining thirteen participants completed the study. All participants missed at least one sample collection during the study either due to travelling or because they were simply unable to provide a sample that day. In total, as seen in fig. 1 two males and 11 females completed the study that originally comprised thirteen female and two male participants.

Food recall and food frequency

The results of the food recall showed that the participants had a wide range of normal fiber intake, with seven grams being the lowest and 30 grams being the highest. The average fiber intake was 19.5 grams, and the average consumption for probiotics was 0.3 foods per day. The food frequency result showed that most participants consumed prebiotic foods with lowest consumption being one prebiotic food a day and highest

being approximately seven per day. Consumption of probiotics was lower. The highest was nine probiotic foods per week and the lowest was zero (table 4).

Log Book

The two-tailed t-test results showed no significance for any of the reported data (table 5 & 8). This was the desired result. The amount of ice cream consumed during the treatment and control was not statistically different and looking at the means show they are very close similar during both periods. The paired samples correlations showed that the change in flatulence level from control to treatment had a strong correlation (0.71) which was statistically significant (0.009). This means that even though the actual level of change was not statistically significant, partially due to sample size, there is a significant level of correlation between when participants were on the treatment and their increase in flatulence level. The same result was shown for number of stools. The paired samples correlation showed number of stools and treatment had a close correlation (0.82) which was significant (0.001). This means that even though the level of overall change was not significant the correlation between the change and the treatment was significant.

Bacteria results; QPCR

The overall statistical analysis for the means during the treatment and control periods showed no statistical significance ($p = 0.11$) but did have a clear difference in means (treatment = 47.13; control = 24.11) (table 3.6). The results of plotting each individual's fold change values over the study were not as expected. The expectation was to see an increase in *B.bifidum* count during treatment and a decrease in *B.bifidum* during washout following the treatment while seeing levels mostly near baseline throughout the rest of the study. The results instead showed spikes during control periods that should not

have been there (table 3.7 parts 1 & 2). Further testing needs to be done to determine exact cause of the inconsistencies. Many difficulties arose with respect to getting the lab procedures to run according to the study plan. Originally, the study design called for making probes specific to the bacteria strains being used.

Conclusion

The synbiotic ice cream did not have any negative impacts on the participants with regard to gastrointestinal symptoms. The results of the bacteria level change were statistically insignificant and further research is needed.

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Figure 1 *B.bifidum* and *L.casei* gel results

The results for the gel electrophoresis that allowed us to select the correct temperature for the *bifidobacterium bifido* primer. The first five sample were the primers with *b.bifidum* with a temperature gradient of 54°C, 56°C, 58°C, 60°C, 62°C. The next five samples were the primers with *Lactobacillus casei* with the same temperature. This showed that the primers did not amplify the other bacteria strain and resulted in choosing 60°C as the annealing temperature. The last 10 samples were the *B.bifidum* and *L.cause* samples with the *L.casei* primer in the same order as the first 10. This illustrated that the primer was not selective enough.

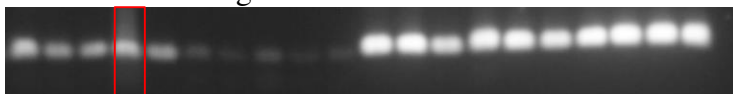


Figure 2 *L.casei* primer test results

The gel electrophoresis for one of the gradient temperature *Lactobacillus casei* primers test. The red boxed area shows the temperature graident of 54°C, 56°C, 58°C, 60°C, 62°C of both *L.casei* primers with *bifidobacterium bifido*. The first five are with primer 4 and second five are with primer 5. The brightly illuminated samples following were 4 different *L.casei* cultures on the same temperature gradient with primer 4 (blue box). The remaining samples were the same 4 *L.casei* cultures on the same gradient with primer 5 (green boxes). These results show that the primers either amplified everything or almost nothing.

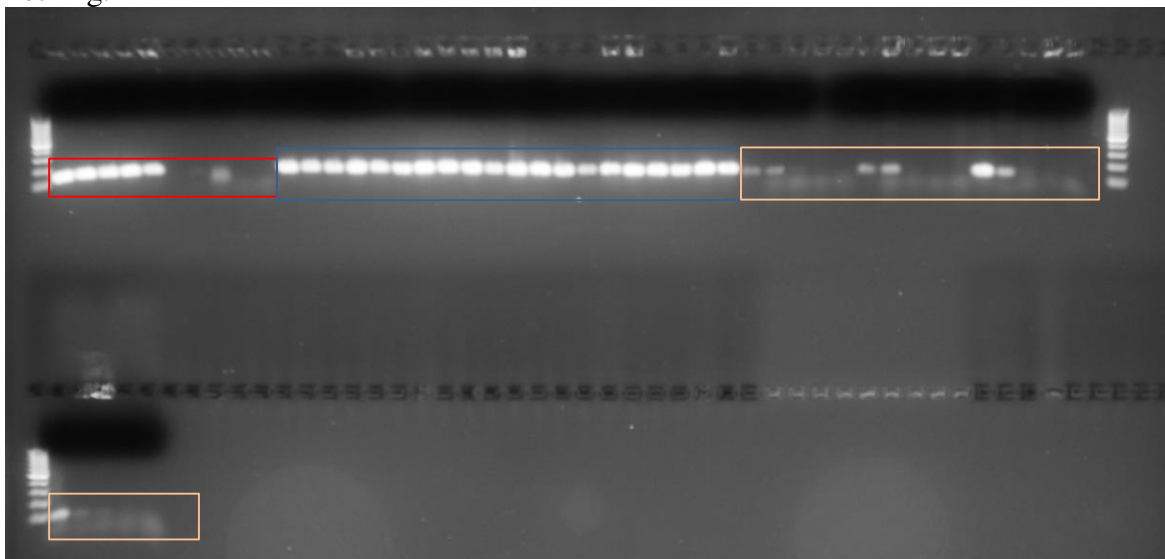


Figure 3: Gender of participants

Participants who completed the study (11 females and 2 males), separated by gender with the numbers representing percentage of males and females.

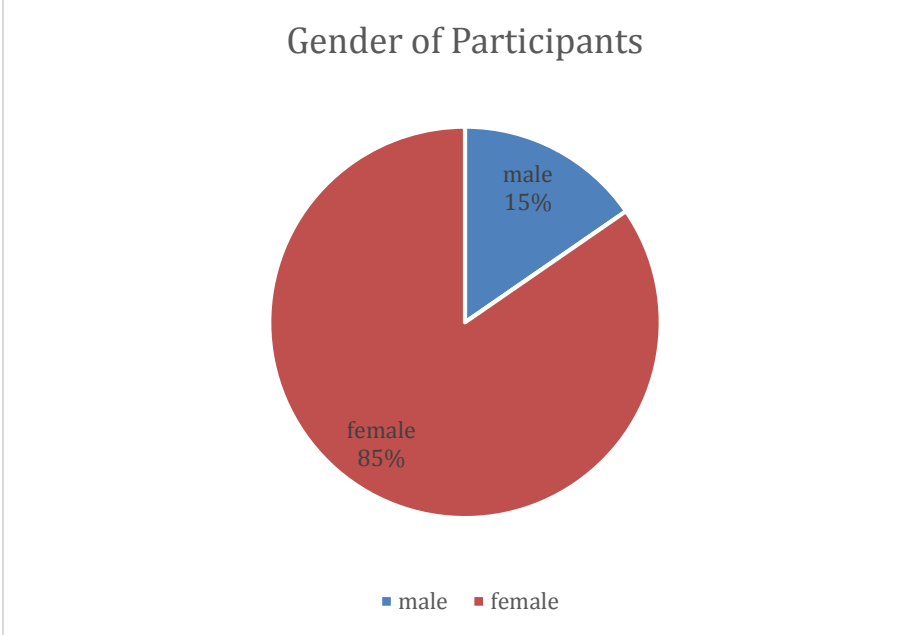


Figure 4: Age of participants

Participant's ages. Showing number of participants of each age with age in years on the y-axis and number of participants that age across.

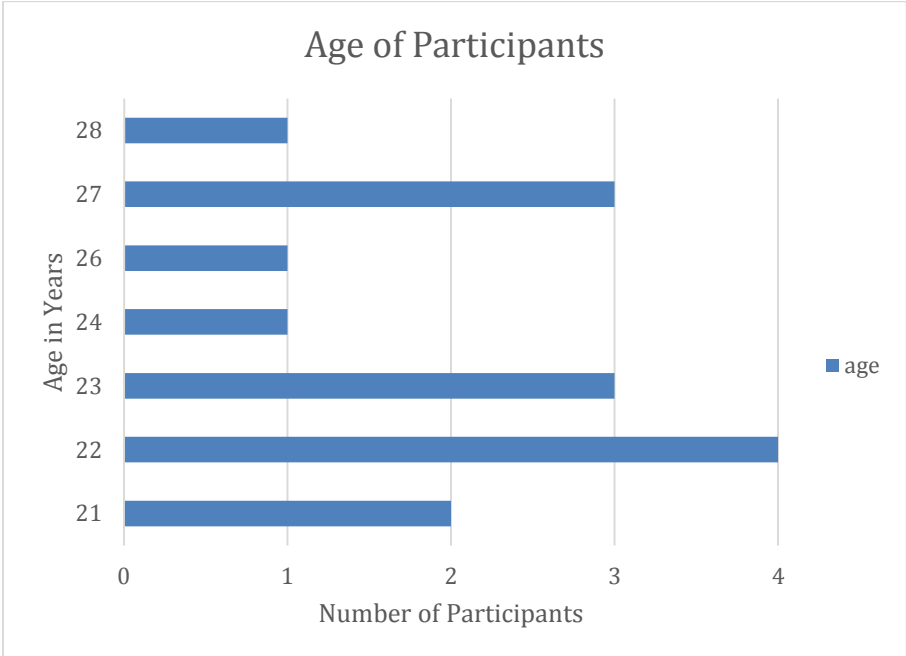


Figure 5: Food Frequency results

Food frequency results for probiotic and prebiotic foods. Showing participants and on far right the average on the x-axis. The number of times they were consumed per week is represented on the y-axis

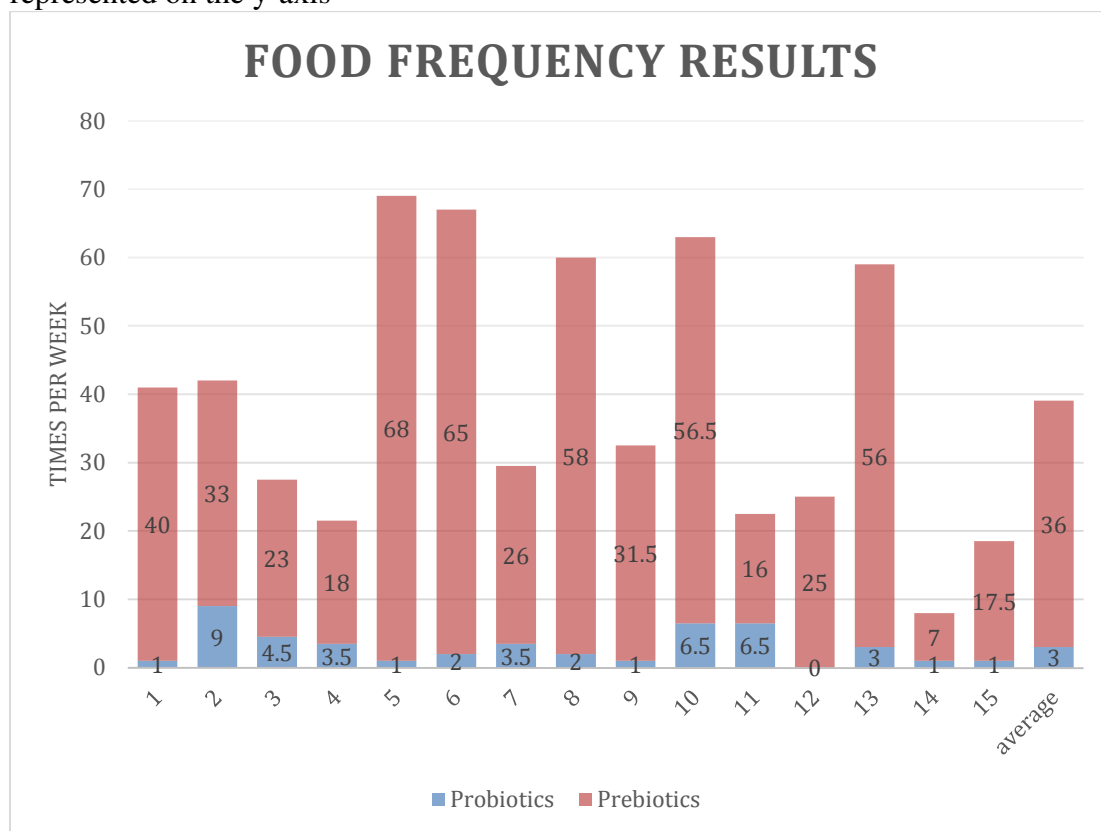


Figure 6: Diet Recall Fiber intake

The average results from each participant's 3-day food recall for daily fiber intake. Each value given number 1-13 represents an individual participant's intake in grams. The final value on the far right of the x-axis is the overall participant average

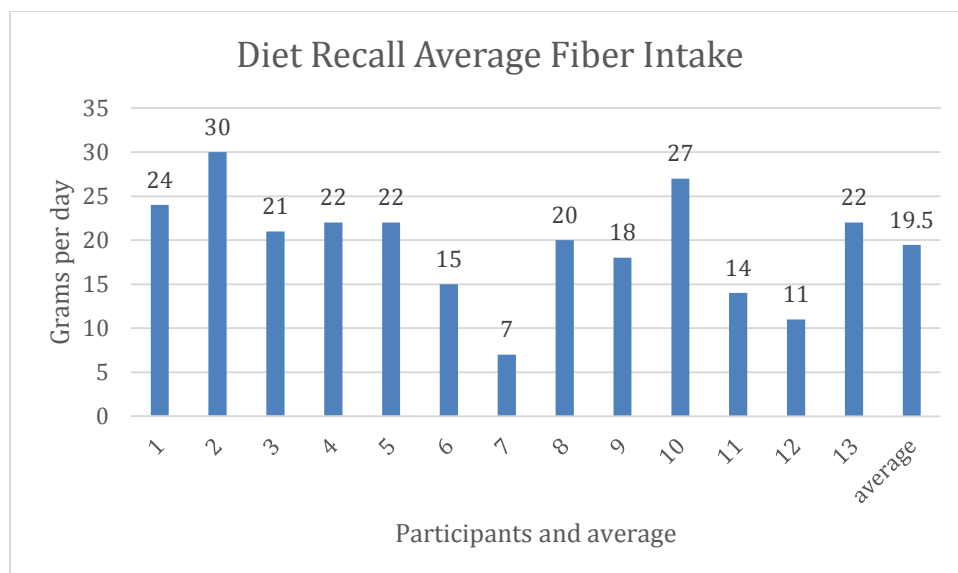


Figure 7: Diet Recall Probiotic Intake

The average results from each participant's 3-day food recall for daily fiber intake. Each value given number 1-13 represents an individual participant's intake in grams. The final value on the far right of the x-axis is the overall participant average

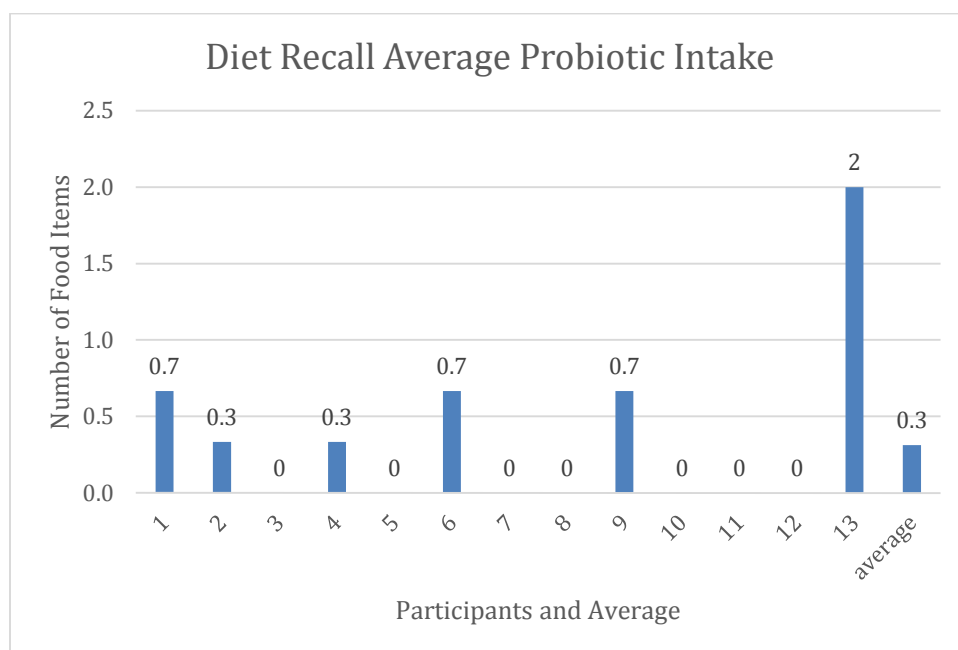


Table 1: Log book t-test results

A two tailed t-test was conducted with a 95% confidence interval and 11 degrees of freedom. The table shows the averages for amount of ice cream consumed, flatulence level, number of stools, and what the Bristol rating for stool consistency was during the control and treatment periods of the study as reported in mean and standard deviation. The mean difference between values during the control and treatment are reported followed by their confidence interval and t-score. All the p values were not significant ($p > 0.05$).

	control (placebo ice cream)	treatment (synbiotic ice cream)	Mean Difference	95% CI	t score	p value
Amount consumed (0%-100%)	92.76 ± 9.74	92.67 ± 9.27	.08929	6.29 to 6.47	.031	0.98
Flatulence (0-10 Likert scale) 0 = none, 10 = constant	3.74 ± 1.56	4.02 ± 1.61	-.28423	1.05 to 0.48	-.817	0.43
Number of Stool (0-10)	1.4 ± 0.51	1.69 ± 0.74	-.26084	0.54 to 0.01	-2.087	0.06
Bristol Rating (0-7) 0 = hard lumps, 7 = liquid	3.18 ± 0.58	3.35 ± 0.82	-.17484	0.72 to 0.37	-.707	0.49

Table 2: B.bifidum qPCR t-test results This was a two tailed t-test conducted with a 95% confidence interval and a degrees of freedom of 12. It shows the fold change averages for *Bifidobacterium bifidum* during the control period of the study and during the synbiotic treatment period. The mean difference is the difference between the two means. The p value was not statistically significant ($p > 0.05$)

	control (placebo ice cream)	treatment (synbiotic ice cream)	Mean Difference	95% CI	t score	p value
<i>B. bifidum</i> fold change	47.13 ± 103.22	24.11 ± 80.29	23.02367	6.12 to 52.16	1.72	0.111

Table 3: B.bifidum qPCR values

The quantified values show the fold change for *B.bifidum* as calculated with the qPCR data for each collection during treatment and washout periods are given. Participants are listed vertically, and data for each participant is listed horizontally across the table.

person	treatment			washout					
	day 7	day 14	day 21	day 1	day 2	day 3	day 7	day 14	day 21
1	0.81	1.15	0.17	0.05		0.35	0.48	0.41	0.00
2	239.11	2.40	1.33	2.71	0.36	5.00	7.42	9.41	3.54
3	0.14	0.08	0.10	0.02	0.08	0.03			5.56
4	1.14		0.26		5.36	0.00	1.15	0.00	0.00
6	474.05	4.01	1.56		0.93	4.15	0.14	0.00	0.76
7	1.24	0.43	0.22	1.39	0.00	0.01	0.15	0.18	2.74
8	0.84	0.81	3.17	0.14	0.73	0.79	0.30	0.16	0.00
9	0.61	3.14	0.53	1.99	0.72	0.44		2.28	1.33
10	6.07		9.11		1.12	1.24	0.07		19.24
12	0.48	0.61	1.45	4.59	0.00		0.13	0.00	492.46
13	0.41	6.96		0.43			1.25	5.95	7.14
14	4.95	0.02	0.07	0.72				3.41	0.11
15	102.66	451.54	504.44	74.74	61.96	74.74	23.33	919.91	77.78

Table 4: B.bifidum qPCR values

The quantified values show the fold change for *B.bifidum* as calculated with the qPCR data for each collection during the control and washout periods are given. Participants are listed vertically, and data for each participant is listed horizontally across the table.

person	control			washout					
	day 7	day 14	day 21	day 1	day 2	day 3	day 7	day 14	day 21
1	0.43		0.32	0.00	0.12	0.01	1.52	1.02	1.21
2	1.39		2.09	0.70	1.69	9.48	0.00	3.70	0.47
3	0.38		2.26	1.62		1.26	0.34	5.87	0.67
4	0.42	1.10	2.37	0.00	0.01	0.03		3.55	4.50
6	1.44	3.90		0.00	0.67		0.10	0.19	
7	0.45		0.51				1.54	0.70	1.42
8	6.89	0.01	3.31	1.97	0.00	0.00	16.77	2.10	0.37
9	0.65	3.45	0.14	1.88	4.54	0.02	1.14	0.66	0.41
10	9.36		0.70				28.76	6.70	
12	2.68	0.08		0.12				0.46	0.06
13	1.79		1.30				1.50	0.78	65.29
14			1.43	0.11	0.37			3.17	0.17
15	830.89	0.00	43.00	139.99	9.58	47.62	110.36	726.05	186.92

Table 5: Log Book correlations

The table shows the correlation between the change in means during the control and treatment periods as well as its significance level for the information reported in the log books.

	Correlation	p value
Amount consumed (0%-100%)	0.443	0.15
Flatulence(0-10 Likert scale) 0 = none, 10 = constant	0.712	0.009
Number of Stool (0-10)	.822	.001
Bristol Rating (0-7) 0 = hard lumps, 7 = liquid	.284	.372

Chapter 4

Implications

Limitations

Participants were allowed to follow their normal diet instead of being put on the same diet to limit confounding factors. As all participants followed their own diet pattern, this could result in statistical inconsistencies. The smaller amounts of prebiotics and probiotics added to the ice cream may have been too small to achieve the desired result. Sample size itself was also a limitation as there were just 13 participants. A larger sample size would have been preferable to determine statistical significance. Purity of the freeze dried bacteria was another limiting factor. Many challenges with the lab analysis were related to issues with the purity level of the bacteria cultures.

When new fresh cultures were isolated from the freeze dried bacteria, their sequencing results came back more contaminated than the previous times. The results for both *Bifidobacterium bifido* and *Lactobacillus casei* came back as matching *Pediococcus acidilactici*. *Pediococcus acidilactici* is a gram-positive cocci that is anaerobic and homofermentative. It is able to grow in a variety of temperatures, pH, and osmotic pressure and is able to colonize the digestive tract. This probiotic is also available from the company where the probiotics used in this study were purchased. Based on the results of the analysis and the difficulty with sequencing the bacteria it seems plausible that the samples were contaminated before delivery. As only the bacteria strains used were being quantified, if the samples did not contain 100% the bacteria stated on the packaging, the probiotic impact could have been lower, limiting our ability to obtain clear results.

Further Research

Based on the results and new research in publication continued research would be more efficacious with cultures that can be proven to be 100% pure. Future studies should try using microencapsulated probiotics to help the bacteria survive through the stomach. To have a better, the quantity of the probiotics and prebiotics added to the ice cream could be increased. A larger sample size would be beneficial in understanding the impact of the synbiotic on the participants. Lastly, instead of examining the changing level of the strains added, examining, the change in the overall microbiome could provide a clearer image of the impact this product has on imparting health benefits on the consumer.

Appendix

Appendix 1. Ice cream formulation and directions followed.

Ice Cream Recipes and directions

Ingredients	Control	Synbiotic W/10% inulin
amounts in grams		
Milk	595	595
Cream	202	202
Nonfat Dry Milk	50	50
Corn Syrup Solids	45	45
Stabilizer	7	7
Sugar	100	90
Inulin	0	10
<i>B.bifidum</i>	0	0.3
<i>Lacto casei</i>	0	0.3

To prepare the ice cream:

1. Dry ingredients were mixed in with the wet ingredients and the ice cream preparation was heated in a microwave during two separate intervals. Inulin is obtained in powder form from the manufacturer SourceNaturals.
2. After 4 minutes, the ice cream preparation was stirred and then put back in the microwave for 3 ½ minutes to solubilize the stabilizer.
3. After heating, the ice cream batch are cooled using an ice bath until they reached a temperature below 40° C so that the probiotics could be at a temperature in which their viability would not compromised. Bifidobacterium bifidum and Lactobacillus casei KE99 were obtained from ProBioFerm in 50 billion colon forming units (CFU) per gram.
4. After adding the probiotics the batch can be poured into an ice cream maker and removed once the ice cream is done churning.

Note: These steps are followed for both recipes minus the addition of the probiotics and prebiotics to the control.

Appendix 3. Recruitment letter**Recruitment Letter**

Hello, my name is Erin McNamara. I am a graduate student working towards my Masters degree in Nutrition and Health Promotion. To further the research of my thesis project, I would like to solicit your help. I am looking for participants who are willing to participate in a 13 week study on the effect of synbiotic ice cream on helpful gut bacteria. The study is for a synbiotic ice cream which contains the prebiotic ingredient inulin (fiber) and probiotics (helpful bacteria) Lactobacillus casei and Bifidobacterium bifido. There will be an initial week where we collect baseline data including 1 stool sample and a 3 day diet diary. Then there will be 2, 3-week treatment periods or feeding periods, where you will consume either the synbiotic ice cream or plain vanilla ice cream. In each of the feeding periods you would consume a ½ cup serving of vanilla ice cream, either the plain vanilla or the synbiotic, once a day and keep a brief journal of different aspects of how you feel. These treatment periods will each be followed by a 3 week wash out period where you follow your normal diet. You will be asked to provide 19 stool samples at different stages in the study so we can track the levels of the two helpful bacteria. Compensation for your efforts would be given upon completion or termination of participation in the study as one lump sum in the form of a Walmart gift card. You will be given \$25 for participation and an additional \$5 per stool sample to total up to \$120. To help further probiotic and prebiotic nutrition research, I ask for your support and participation in this study. For more information and to participate please use the below contact information.

Thank you,

Erin McNamara
University of Nebraska - Lincoln
Department of Nutrition and Health Sciences
Lincoln, NE 68583-0806
Phone: 402-206-1433
Email: erinmm88@gmail.com

Appendix 4. Screening Questionnaire

Screening questionnaire

- | | | | |
|--|-----|----|-----------|
| 1. Are you over 19 years of age? | Yes | No | |
| 2. Are you female | Yes | No | |
| If yes, are you currently pregnant or breastfeeding? | Yes | No | |
| If yes, are you post menopausal? | Yes | No | |
| 3. Do you have chronic diarrhea or constipation? | Yes | No | |
| 4. Do you have diverticulosis or diverticulitis? | Yes | No | |
| 5. Do you currently consume probiotics? | Yes | No | |
| If yes how often? | | | |
| 6. Do you currently consume prebiotics? | Yes | No | |
| If yes how often? | | | |
| 7. Are you currently or have recently taken antibiotics? | Yes | No | |
| If so when and for how long? | | | |
| 8. Were you breastfed as an infant? | Yes | No | Uncertain |
| If yes for how long? | | | |

Appendix 5. Letter of informed consent



College of Education and Human Sciences
Department of Nutrition and Health Sciences

Informed Consent Form

Consent Form for Participation in a Research Study

Title of Research Project: The effect of Synbiotic ice cream on gut bacteria viability in the GI tract

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Introduction:

You are invited to participate in a research study to determine the effect of synbiotic ice cream on gut bacteria viability. Synbiotic dietary supplements are made of prebiotics—fiber--combined with probiotics--helpful bacteria. This consent form will give you the information you will need to understand why this study is being done and why you are being invited to participate. It will also describe what you will need to do to participate and any known risks, inconveniences or discomforts that you may have while participating. You are encouraged to think this over. You are also encouraged to ask questions now and at any time. If you decide to participate, you will be asked to sign this form and it will be a record of your agreement to participate. This process is called 'informed consent.' You will receive a copy of this form for your records

Purpose of the study:

The purpose of this study is to determine if regular consumption of ice cream containing a synbiotic blend of fiber, inulin, and helpful bacteria--Bifidobacterium bifidum and Lactobacillus casei--will increase the levels of these two helpful bacteria in the gut.

Procedures:

If you agree to participate in this study, you will be asked to do the following:

- Complete a screening form to see if you qualify for the study. You are invited to participate if you are a healthy adult with no history of colon diseases, diverticulosis/diverticulitis, no chronic diarrhea or constipation, and have not been on antibiotics in the last 6 weeks. If you are a women you must be premenopausal and not pregnant or breastfeeding.
- Prior to starting the study (week 0), you will be asked to fill out a 3 day diet diary on 3 consecutive days to give us an idea of your normal diet.
- Weeks 1-3 is the first feeding period, you will consume ½ cup portion of ice cream daily. The ice cream will either be plain vanilla, made using the UNL dairy store recipe, or this same ice cream with two types of helpful bacteria (like those found in yogurt),

Lactobacillus casei and *Bifido bacterium bifido*, and one prebiotic, inulin (a type of fiber) added. Each day you will fill out a brief log book evaluating your stool and gas levels as well as tracking how much ice cream you consumed.

- At the end of each week of the study (days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84) you will be asked to provide a stool sample (at least 1 gram is needed). This sample will need to be collected by you then either brought directly to campus or kept chilled or frozen until it can be dropped off in the provided coolers. By using the coolers, you do not have to bring the samples immediately to campus but can bring them at a time that works best for you. An email reminder to collect each sample and schedule a drop off time will be sent to you or you can email the primary investigator. All needed equipment will be provided, including stool sample collectors, gloves, and coolers.
- Weeks 4-6 is the first washout period. You will be asked to follow your normal diet and collect stool samples on each day for the first 3 days (days 22, 23, 24) of week 4 as well as the above mentioned weekly samples. These samples are needed because often the most rapid change in number of the helpful bacteria occurs at this time.
- Weeks 7-9 is the second feeding period and you will do the same process as described for weeks 1-3.
- Weeks 10-12 is the second washout period. The steps will be the same as week 4-6 with samples collected on the first 3 days (days 64, 65, 66).
- Email reminders will be sent throughout the study to remind you to collect stool samples, schedule drop off times, and to schedule times for you to pick up your ice cream. The meeting points will either be Ruth Leverton Hall room 115 or 312.
- Not everyone has a bowel movement every day. If there is a day that you cannot provide a sample you can still continue your participation in the study. If this becomes a frequent issue, you may want to discuss discontinuing participation with the primary investigator. Also, be aware that each sample you miss will decrease your compensation received at the end of the study.

Benefits:

There are no direct benefits to participants. They are contributing to furthering scientific research which could benefit society through the development of a product that may promote gut health improvements.

Risks and/or Discomforts:

Participants may experience some discomfort due to gas, bloating, constipation, or diarrhea as a side effect but this usually goes away in a few days and is the normal reaction of the body to increasing fiber intake. If any problems arise you should seek medical care at your medical care provider at your own expense.

Confidentiality:

Any information obtained during this study which could identify you will be kept strictly confidential. Documents linking your name to your assigned numeric code will be destroyed after all data has been collected and verified. The other paper data (all those with just your numeric code) will be stored in a locked cabinet in LEV 312 and will only be seen by the investigators during the study and will be stored for the required 3 years. All stool samples will be stored in LEV 114 or ASCI C120 using the numerical code you will be assigned and disposed of after analysis. The information obtained in this study

Appendix 6. Food Frequency Form

Probiotics	less than 1 a week	Once a week	2-3x a week	4-6x a week	once a day	2+ a day
Kefir						
Yogurt						
Milk with added live cultures						
Buttermilk						
Smoothies, butter, and sour cream which are labeled "cultured"						
Fermented foods like saurkraut, kimchi, or tempeh						
Miso						
Microalgae ex/spirulina, green algae						
Probiotic supplements (pills, powder, etc.)						
Prebiotic	less than 1 a week	Once a week	2-3x a week	4-6x a week	once a day	2+ a day
How often do you eat any fruit fresh or canned (not counting juice?)						
Fruit juice like orange, apple, grape; fresh, frozen, or canned. (not sodas or other drinks?)						
Vegetable juice, like tomato juice, V-8, carrot						
Green salad						
Potatoes, any kind, including baked, mashed, or french fried						
Vegetable soup, or stew with vegetables						
Any other vegetables, including green beans, peas, corn, broccoli, or any other kind						
Fiber cereals like raisin bran, shredded wheat or Fruit-n-Fiber						
Beans such as baked beans, pinto, kidney, or lentils (not green beans)						
bread such as whole wheat, white whole wheat or rye						

Appendix 7. Daily log book page

Daily Log

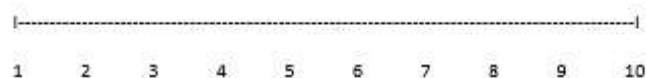
An entry is to be made every day during ingestion periods 1 and 2.

Date:

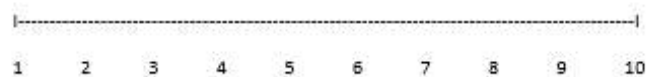
Amount of ice cream consumed



Flatulence Frequency



Stool frequency



Stool Consistency: circle all that apply

Bristol Stool Chart

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on its surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges (passed easily)
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. Entirely Liquid

Comments or additional info:

Appendix 8.

Weekly schedule for study with fecal collection days highlighted in blue.

Phase	Day of Study						
Prefeeding							0
Feeding	1	2	3	4	5	6	7
	8	9	10	11	12	13	14
	15	16	17	18	19	20	21
Washout	22	23	24	25	26	27	28
	29	30	31	32	33	34	35
	36	37	38	39	40	41	42
Feeding	43	44	45	46	47	48	49
	50	51	52	53	54	55	56
	57	58	59	60	61	62	63
Washout	64	65	66	67	68	69	70
	71	72	73	74	75	76	77
	78	79	80	81	82	83	84

Appendix 9. USDA supertracker example section from multi-day report

Nutrients Report

Your plan is based on a default 2000 Calorie allowance.

Nutrients	Target	Average Eaten	Status
Total Calories	2000 Calories	1712 Calories	OK
Protein (g)***	46 g	79 g	OK
Protein (% Calories)***	10 - 35% Calories	19% Calories	OK
Carbohydrate (g)***	130 g	228 g	OK
Carbohydrate (% Calories)***	45 - 65% Calories	53% Calories	OK
Dietary Fiber	25 g	24 g	Under
Total Sugars	No Daily Target or Limit	64 g	No Daily Target or Limit
Added Sugars	No Daily Target or Limit	15 g	No Daily Target or Limit
Total Fat	20 - 35% Calories	30% Calories	OK
Saturated Fat	< 10% Calories	7% Calories	OK
Polyunsaturated Fat	No Daily Target or Limit	7% Calories	No Daily Target or Limit
Monounsaturated Fat	No Daily Target or Limit	13% Calories	No Daily Target or Limit
Linoleic Acid (g)***	12 g	12 g	OK
Linoleic Acid (% Calories)***	5 - 10% Calories	6% Calories	OK
α -Linolenic Acid (% Calories)***	0.6 - 1.2% Calories	0.3% Calories	Under
α -Linolenic Acid (g)***	1.1 g	0.6 g	Under
Omega 3 - EPA	No Daily Target or Limit	9 mg	No Daily Target or Limit
Omega 3 - DHA	No Daily Target or Limit	34 mg	No Daily Target or Limit
Cholesterol	< 300 mg	171 mg	OK

Appendix 10. example page section from daily SuperTracker



View by Meal

[Print Page](#)

Meals at a glance! See the food group amounts eaten at each meal.

Your Day

Breakfast Totals	Lunch Totals	Dinner Totals	Snack Totals
Grains 0 oz.	Grains 3 oz.	Grains 6 oz.	Grains 0 oz.
Vegetables 0 cup(s)	Vegetables ½ cup(s)	Vegetables ½ cup(s)	Vegetables 0 cup(s)
Fruits 0 cup(s)	Fruits 0 cup(s)	Fruits 0 cup(s)	Fruits 1 cup(s)
Dairy 0 cup(s)	Dairy ½ cup(s)	Dairy 0 cup(s)	Dairy 1 cup(s)
Protein Foods 1½ oz.	Protein Foods 0 oz.	Protein Foods 0 oz.	Protein Foods 1 oz.
Oils 0 tsp.	Oils 3 tsp.	Oils 2 tsp.	Oils 1 tsp.
Breakfast 1 large egg(s) Scrambled eggs (no milk added), cooked with nonslick spray 1 ounce(s) Protein Foods 2 medium slice Bacon, pork, cooked ½ ounce(s) Protein Foods ½ mug (8 fl oz) Coffee, brewed, regular (no food groups)	Lunch 1½ cup Quinoa, cooked 3 ounce(s) Grains 1 tablespoon Oil, olive 3 teaspoon Oils 1 teaspoon Lemon juice, canned or bottled (no food groups) ½ cup, cherry tomatoes Tomato, cherry ½ cup(s) Vegetables ½ cup, shredded Mozzarella cheese, part skim ½ cup(s) Dairy 1½ cup (8 fl oz) Water, tap (no food groups)	Dinner 2 cup Rice, brown, regular, cooked (no salt or fat added) 4 ounce(s) Grains ½ cup Pinto beans, canned (no fat added) ½ cup(s) Vegetables ½ cup Corn, yellow, canned, cooked, no fat added ½ cup(s) Vegetables 1 tablespoon Cilantro, raw (no food groups) 1 tablespoon, chopped Pepper, jalapeno (no food groups) 8 chip(s) Tortilla chips (Tostitos, Doritos) 1 ounce(s) Grains 1 teaspoon Oils 7 chip(s) Tortilla chips (Tostitos, Doritos) 1 ounce(s) Grains 1 teaspoon Oils 2 cup (8 fl oz) Water, tap (no food groups)	Snack ½ cup Mango, dried ½ cup(s) Fruits ½ medium (7" to 7-7/8" long) Banana, raw ½ cup(s) Fruits 1 tablespoon Peanut butter, reduced sugar 1 ounce(s) Protein Foods 1 teaspoon Oils 1 cup 1% milk 1 cup(s) Dairy
Food Groups	My Daily Target	Enter	How Did I Do?

Appendix 11

Sequences for primers used:

Forward: 5'-GAG TGT ACC TTT CGA ATA AGC-3'

Reverse: 5'-CCC TTT ACG AAT AAA TC-3'

Appendix 12 dissociation curve results for 16s set 1 example image with boxed part being one of the samples that was thrown out for inaccuracy.

