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Title: Mechanisms of Preventing Antibiotic-Associated Diarrhea and the Role for Probiotics

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CLINICAL RESEARCH PROTOCOL

Exploratory Pilot Studies to Demonstrate Mechanisms of Preventing Antibiotic-Associated Diarrhea and the Role for Probiotics

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Sponsor of IND:

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Signature Page

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and International Conference on Harmonisation guidelines.

Principal Investigator: Daniel Merenstein, MD

Signed: 

Daniel Merenstein, MD
Principal Investigator

Date: July 28, 2021

Statement of Compliance

The study will be carried out in accordance with Good Clinical Practices (GCP) as required by the following:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46; 21 CFR Part 50, 21 CFR Part 56, and 21 CFR Part 312)
- ICH E6; 62 Federal Register 25691 (1997)
- NIH Clinical Terms of Award

All key personnel (all individuals responsible for the design and conduct of this study) have completed Human Subjects Research Protection Training.

STUDY TEAM ROSTER

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PRÉCIS

Study Title: Exploratory Pilot Studies to Demonstrate Mechanisms of Preventing Antibiotic- Associated Diarrhea and the Role for Probiotics

Phase: Mechanistic Study

Population: 60 healthy adults ages 18-65 years

Number of Sites: Single Center

Study Duration: R61 Phase: 24 months

Description of Study Design: Randomized, double-blinded, controlled clinical trial

National Clinical Trial (NCT) Number: NCT03755765

Georgetown University Institutional Review Number: 2018-0736

Primary Objective:

To determine the ability of BB-12 to impact antibiotic-induced reduction in SCFA concentration as reflected by the levels of acetate on day 7. SCFA produced by anaerobic gut microbiota will be quantified from human fecal samples via liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Secondary Objective:

To determine the ability of BB-12 to impact antibiotic-induced disruption of the gut microbiota with 16S rDNA profiling. This will be assessed at days 7 and 14. Changes in absolute abundance of specific bacterial genera (with a particular emphasis on known butyrate producers in the Firmicutes phylum) will also be assessed with qPCR.

Design and Outcomes:

This is a randomized, double-blinded, single center, mechanistic study to test the mechanism of Bifidobacterium animalis subsp. lactis (B. lactis) strain BB-12 (BB-12) yogurt in AAD, compared to yogurt without BB-12, in healthy adults. Sixty participants will be randomized to two groups, BB-12 supplemented yogurt (40) and control yogurt (20), using a 2:1 random allocation rule. All participants will be given a 7-day prescription for 875/125 mg of amoxicillin-clavulanate (AMC), taken twice daily. Participants will be recruited through the Capital Area Primary Care Research Network (CAPRICORN), a practice-based research network (PBRN), and other community sites.

Interventions and Duration:

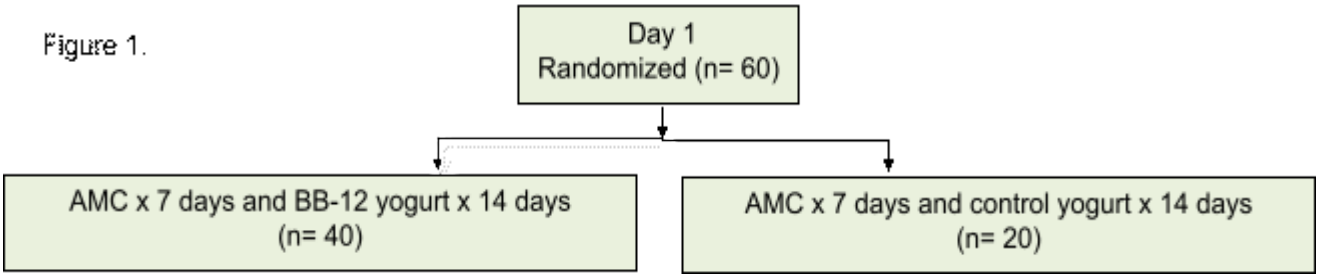
The probiotic-supplemented yogurt is composed of the investigational agent, BB-12, and contains the yogurt starter culture YF-L702 (a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*). The yogurt starter culture will be present and alive in the control yogurt. Participants are to provide informed consent at Baseline and complete a 30-day run-in period, during which they will refrain from all probiotics, and begin the antibiotic and yogurt interventions on Day 1.

Participants will be randomized to receive BB-12-supplemented yogurt or control yogurt (without BB-12), in a 2:1 (40:20 participants) allocation (**Figure 1**).

Group 1 (n=40): Probiotic, BB-12-supplemented yogurt, 4-ounces (which delivers $\geq 10^{10}$ CFU of BB-12) taken orally for 14 days and amoxicillin-clavulanate 875/125 mg, twice daily, for 7 days.

Group 2 (n=20): Control yogurt, 4-ounces taken orally for 14 days and amoxicillin-clavulanate 875/125 mg, twice daily, for 7 days.

Figure 1.



Fecal samples will be collected at home (at baseline (pre and post run-in), days 7, 14, 21 and 30) for SCFA and microbiome testing. Participants will be given detailed, written and illustrated instructions on proper sample collection procedures, and research staff will review these protocols with participants during enrollment.

Follow-up phone visits will be completed on Days 7, 14, and 30. Participants will keep a daily diary to track number, shape and consistency of bowel movements, if/when antibiotic and/or yogurt was consumed, Bristol Stool chart, general health, use of other medicines or products, adverse events, and the Gastrointestinal Symptom Rating Scale (GSRS) - Irritable Bowel Syndrome (IBS) version..

Duration: Each participant will be in the study for approximately 8 weeks total. During the first 30 days, the participant will refrain from antibiotics and probiotics (run-in period) and provide two baseline samples. The participant will be taking the antibiotic intervention for 7 days and taking the yogurt intervention for 14 days. There are two more data collection visits on day 21 and 30.

Pre run-in Baseline	Run-in (30 days)	Post run-in Day 0	Day 1	Day 7	Day 14	Day 21	Day 30
Informed Consent Inclusion/ exclusion Enrollment Demographics Baseline health GSRS-IBS Fecal sample	No antibiotics No probiotics Fecal sample	Fecal sample Post-run in inclusion/ exclusion Post run-in health status Randomization	AMC start Yogurt start Diary start	AMC end GSRS-IBS Fecal sample Follow-up	Yogurt end Diary end GSRS-IBS Fecal sample Follow-up	Fecal sample	Fecal sample Follow-up

Sample Size and Population:

The population will be generally healthy adults of ages 18-65 years. This randomized controlled trial is open to all eligible adults who meet the inclusion criteria. We are not proposing exclusion of any specific sex/gender or racial/ethnic group. One hundred participants will be recruited from the greater community and clinical sites in the Washington, DC metropolitan area, and sixty (n=60) participants will be randomized and commence the study. Based on the demographics of the network and recruitment rates in previous studies at our site, we anticipate about 50% of participants will be women, 20% will be Spanish speakers, and 30-40% will self-identify as one of the racial or ethnic minority populations.

1. STUDY OBJECTIVES

1.1 Primary Objective

To determine the ability of BB-12 to impact antibiotic-induced reduction in SCFA concentration as reflected by the levels of acetate on day 7. SCFA produced by anaerobic gut microbiota will be quantified from human fecal samples via liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Primary Hypothesis: Antibiotics will result in a reduction in fecal SCFA by day 7. BB-12 supplementation will protect against antibiotic-induced SCFA reduction and/or be associated with a quicker return to baseline SCFA levels, as compared to the control group.

1.2 Secondary Objectives

To determine the ability of BB-12 to impact antibiotic-induced disruption of the gut microbiota with 16S rDNA profiling. This will be assessed at days 7 and 14. Changes in absolute abundance of specific bacterial genera (with a particular emphasis on known butyrate producers in the Firmicutes phylum) will also be assessed with qPCR.

Secondary Hypothesis: Antibiotics will result in a decrease in the overall number and diversity of bacterial species present in the fecal microbiota. This will be assessed at baseline and days 7, 14, 21 and 30, and we would expect a decrease in at least days 7 and 14. BB-12 supplementation will protect against antibiotic-induced shifts in the microbiota and/or will be associated with a quicker return to a baseline microbiota composition, as compared to the control group. Microbiota composition will be assessed with 16S rDNA profiling and qPCR.

2. BACKGROUND AND RATIONALE

2.1 Background Information

Probiotics are live microorganisms that, when administered in sufficient amounts, may improve health.(1) Often, probiotics are ingested as supplements in powder, pill or liquid forms, designed specifically for medicinal benefit. Such supplements have shown potential benefits in treatment and prevention of varied diseases, including diarrhea, asthma, necrotizing enterocolitis and allergies.(2-6) As an alternative to supplements, probiotics are also included as ingredients in fermented dairy products to produce functional foods; that is foods providing health benefits beyond their nutritional value.(7-10) Yogurt, for example, is a fermented milk product often considered a functional food. In fact, two-thirds of primary care physicians who counsel patients about nutrition recommend consuming yogurt containing live active cultures for the health benefits associated with this food.(11) Despite this common practice, however, evidence for beneficial health outcomes is limited.

2.2 Microbiota Play an Important Role in the Gastrointestinal Tract

The gastrointestinal tract contains a complex commensal microbiota that contributes to homeostasis in the gastrointestinal tract. The intestinal microbiota includes hundreds of species of facultative and obligate anaerobes.(12) The human intestine is home to over 100 trillion microorganisms. When functioning appropriately, this high-species diversity produces homeostasis of the gut.(13) This balance is often disturbed by medical interventions such as antibiotics or viral diseases, causing a disturbance of the fecal microbiota and resulting in, among other effects, decreased short chain fatty acid metabolism. This causes the accumulation of luminal carbohydrate, subsequent pH changes, and water absorption.(14) Supplemental probiotics may help regulate the microbiota when disturbed by outside influences.(15)

2.3 General *Bifidobacterium* Information

Probiotics marketed as nutritional supplements and found in functional foods, such as yogurts, are principally members of the genera *Bifidobacterium* and *Lactobacillus*. Members of these genera are Gram-positive facultative anaerobes and are also classified as lactic acid bacteria (LAB). *Bifidobacterium* species, particularly *B. lactis* BB-12, the principal focus of this study, can be found in the gastrointestinal tract (GIT) as both autochthonous (native to a particular place) and allochthonous (derived from outside a system) residents.(16) Newborns, especially those that are breast-fed, are colonized with bifidobacteria within days after birth. Once the child is weaned, the population of these bacteria in the colon appears to be relatively stable until advanced age when it appears to decline.(16, 17) Furthermore, *B. lactis*, unlike the majority of anaerobic bifidobacteria, are moderately tolerant of oxygen. It is for this reason that *B. lactis* is so extensively used for research and commercial application.(18)

2.4 Likely Mechanisms of BB-12 Health Effects

There are many potential mechanisms by which probiotics are believed to exert their effects. Possible mechanisms of probiotic effect include: 1) production of antimicrobial substances; 2) binding to and penetrating gastrointestinal receptors; 3) competition for nutrients; 4) enhancement of mucosal barrier function; 5) altered immunoregulation, including both anti-inflammatory and immunostimulatory responses; and, 6) ferment glucose, lactose, and fructose, thereby lowering fecal pH.(19-25)

Due to the immune system's role in a multitude of disease states, many studies have concentrated on the immunoregulatory effect of probiotics, examining probiotics' role in diseases, such as eczema with potential mechanisms involving T cell, B cell, epithelial cell, dendritic cell, macrophage, nature killer cell, antibody, and cytokine effects.(26-28) After providing infants who manifested atopic eczema (mean age

of 4.6 months) with a BB-12 infused formula, Isolauri, et al., found significant skin improvements in the probiotic group compared to the control group (P=0.002).(28)

Additionally, many believe that inflammatory bowel disease (IBD) appears to be caused by immune responses to commensal enteric bacteria in predisposed individuals.(24) A commercial blend of eight different probiotic strains (Streptococcus thermophilus, three strains of *Bifidobacterium* species and four strains of *Lactobacillus* species), VSL3, has been shown to limit epithelial inflammatory responses in vivo and in vitro, and is often prescribed for individuals with IBD.(29-34)

Further support of an immunoregulatory response is a study conducted by Fukushima et al. on healthy Japanese children between the ages of 15 and 31 months. The children were fed a follow-up formula infused with 10^9 CFU BB-12 and were found to have a significant increase in total IgA and anti-poliovirus IgA, respectively.(35) This suggests ingestion of the probiotic formula containing BB-12 and colonization by the strain can trigger IgA production by the host. This type of data demonstrates that probiotics positively influence the maturation process of immunity.

2.5 BB-12 Survival in Stools

The ability to isolate an orally administered probiotic in the stools is considered to be a good indicator that the strain is able to reach key target sites throughout the intestinal tract. The ability of BB-12 to survive intestinal transit has been documented in several studies.(36-39) Taken together, these studies document the ability of BB-12 to survive gastrointestinal transit and persist for 1-3 weeks post feeding. However, the degree of persistence is likely due to doses fed, delivery vehicle, host factors and the ability of BB-12 to grow during colonization. Studies such as the one proposed here are needed to document the success of probiotic survival in foods supplemented with probiotics.

2.6 Safety of Use of BB-12

Lactic acid bacteria (LAB) in foods have a long history of safe use. LAB and related organisms (with the exception of enterococci) are seldom associated with infections, except in immunodeficient individuals or individuals with severe underlying illness.(40) Although bifidobacteria are not naturally found in food, they have been used as ingredients of dietary supplements and added to foods as functional ingredients for decades.(16) The BB-12 strain has been used in numerous controlled human studies in pediatric and adult populations with no adverse incidents reported. BB-12, in combination with a strain of *S. thermophilus*, was the subject of a GRAS petition to the FDA March 2002 (Agency GRAS Notice GRN 000049), where the FDA did not object to use of these bacteria in infant formula.

Although transferable antibiotic resistances are not very common among LAB, they do occur. BB-12 was evaluated for such resistance genes and was found to contain the tetracycline resistance gene *tet*(W). Nestlé provided these data to the FDA in support of its view that the presence of *tet*(W) does not affect the safety of BB-12 as an ingredient in infant formula. In November 2005, the FDA responded to this additional information stating that:

> "Based on the information provided by Nestlé, as well as other information available to FDA, the agency has no questions at this time regarding Nestlé's conclusions that the presence of the *tet*(W) gene in their *B. lactis* strain does not affect the safety of the intended use of *B. lactis* as an ingredient in infant formula and that the discovery of the *tet*(W) gene in *B. lactis* does not change their previous conclusion that *B. lactis* is GRAS for its intended use as an ingredient in infant formula."

A summary of the BB-12 safety articles, using the identical strain as in this protocol, is included in the **Appendix**.

2.7 Probiotics Treating and Preventing Antibiotic-Associated Diarrhea (AAD)

Acute diarrhea is commonly caused by infections or antibiotics. Children and adults are often placed on antibiotics and the rate of diarrhea associated with antibiotic usage is 20-35%, with 10-20% of the diarrhea due to *Clostridium difficile*.(41-49)

Antimicrobial resistance, stemming from antibiotic use, is a multifactorial problem. Major elements of the problem include over-prescription of antibiotics by physicians and poor compliance by patients.(50) Although educating physicians and the lay public has led to dramatic decreases in antibiotic prescriptions, approximately 25% of all visits for children under age five still results in a prescription for antibiotics.(48, 49, 51-53) In fact, children ages three to 36 months average over two antibiotic prescriptions per year, with nearly 30% receiving over four prescriptions per year.(49)

Probiotics have the potential to reduce the rate of AAD. Numerous meta-analyses have examined the potential role of probiotics, with the positive studies showing an effect size of 25-35%.(54-59) In a recent study, Correa et al. enrolled 80 children between six and 36 months of age who were receiving oral or parental antibiotic therapy. In addition to the antibiotics, infants were given either a combination of 10^7 *B.lactis* and 10^6 *S. thermophilus* or a placebo. In this study, 31% of the placebo group experienced diarrhea compared to 16% of the probiotic group (p, 0.044).(60) In a similar study, Arvola et al enrolled children on antibiotics and gave them either a matching placebo or LGG at $2x10^{10}$ CFU/day. After two weeks, 5% of the LGG group had diarrhea compared to 16% of the placebo group, with a treatment effect of -11% (95% confidence intervals of -21%, 0%).(61)

2.8 Functional Foods and Yogurt

Consuming foods to provide health benefits beyond basic nutritional needs is a well-accepted practice in the United States. For example, many parents feed their children cereal fortified with minerals and nutrients. Another example is the addition of folic acid to many food products; this has greatly decreased the incidence of neural tube defects in the U.S.(62, 63) Functional foods are enriched or enhanced foods that provide health benefits beyond the inherent nutritional value of the food.(64) For example, BENECOL® is a margarine type food proven in clinical trials to reduce LDL cholesterol.(65, 66) The promise of using functional foods to mitigate disease and promote health is one of the major reasons so many resources are being devoted to this exciting new field.(9, 67-70)

Yogurt is defined as milk fermented with two specific bacterial species, *L. bulgaricus* and *S. thermophilus*. To differentiate yogurt containing live and active cultures and those pasteurized after fermentation, the National Yogurt Association developed a seal, the Live Active Culture Seal. Any yogurt containing 10^8 CFU/g at the time of manufacture may display this seal. A dose of probiotics of 10^8-10^{10} cfu/day is the amount that has generally been studied and has resulted in health benefits. However, several surveys suggest the probiotic content of yogurt and probiotic supplements may be lower than labels would imply. Hamilton-Miller et al. examined 52 European probiotic products, including 11 yogurts, and found that none gave any indication of actual numbers and less than 50% accurately stated the bacterial species contained in the product.(71) This study highlights the lack of useful information available to consumers when selecting products containing probiotics.

Beniwal et al. studied the effect of yogurt containing *L. acidophilus* on AAD in an unblinded manner in hospitalized patients receiving oral or intravenous antibiotics. The 105 patients in the yogurt group received two 8-ounce vanilla yogurt containers for eight days. Yogurt supplemented with *L. acidophilus* decreased AAD from 24% to 12%.(72) However, the unblinded nature of this study makes these results only suggestive.

2.9 Summary and Significance

Bifidobacterium lactis strain BB-12 is a commercially available probiotic strain that has been used in a number of feeding and clinical trials.(73-84) BB-12 has been found to survive transit through the stomach, small intestine and colon.(85-87) Additionally, long term consumption of probiotic-containing formula at levels as high as 1 billion cfu/g (240 g serving) was found to be safe and well tolerated by children.(88, 89)

Along with providing health benefits, BB-12 is a highly sought probiotic because it has a high resistance to acids in fermented dairy products and is aerotolerant.(90) Importantly for our study, it is believed that dairy products provide a protective environment allowing for probiotic bacteria to remain viable through digestion.(90) An issue concerning probiotic delivery in dairy products is a decrease in viable cells by the end of the product's shelf-life. McBearty et al. manufactured a probiotic cheddar cheese containing 10^8 cfu/ml BB-12.(91) The levels of BB-12 remained constant throughout six months of ripening at a pH of 5.3. Fukushima et al. incorporated BB-12 into an unfermented, canned infant formula.(35) The levels of viable BB-12 cells did not decrease for at least 18 months in the sealed can and for 30 days after the can was opened. BB-12 appears to persist in dairy products; however, the persistence of BB-12 in yogurt, where the pH is lower than 5.0, has not been as widely studied.

Nearly 190 million outpatient antibiotic prescriptions were given in the period of 1998-99. Nearly one quarter (22%) of all pediatric visits resulted in antibiotic prescriptions. A study of 1992 data reported that office visits for colds, upper respiratory tract infections, and bronchitis resulted in approximately 12 million antibiotic prescriptions, accounting for 21% of antibiotic prescriptions.(92) Another study found that antibiotics were prescribed for 78% of acute bronchitis episodes, 65% of acute pharyngitis episodes, 81% of acute sinusitis episodes, and 33% of nonspecific URI episodes, many of which are of viral etiology.(93)

Noncompliance with prescriptions is often due to side effects, most commonly AAD.(94) Diarrhea is a common and costly disease of children in the U.S. Children less than five years of age experience 20-35 million episodes of diarrhea per year. These episodes lead to 2-3.5 million physician visits (which account for 10% of all visits by children), more than 200,000 hospitalizations (13% of hospital admissions in children less than five years), and 325-425 deaths annually.(95-98) There have been many promising studies demonstrating the potential benefits of probiotic supplementation in preventing diarrhea. However, currently, probiotics are generally given in powder, pill or liquid forms and generally sold at health food stores. Yogurt, which is more readily available and accepted, has the potential to greatly influence health if efficacious probiotic dosages and species are available in the product.

This study will evaluate whether a well-defined, probiotic-containing yogurt can be used as an effective delivery vehicle for probiotics. Our long-term goal is to create yogurt with sufficient probiotic dosages to positively impact many different aspects of childhood and adult health. There is widespread acceptance of yogurt with added cultures among the general population and this is one of the reasons we believe yogurt will be more acceptable than supplements.

2.10 Summary of Completed Studies

***I. Safety of BB-12 Supplemented Strawberry Yogurt For Healthy Adults on Antibiotics*:** The primary objective of this Phase 1 study was to establish safety of probiotic BB-12 supplemented yogurt consumed daily for 10 consecutive days by adults concurrently taking antibiotics for an upper respiratory infection. Forty subjects were randomized into two groups, BB-12 supplemented yogurt and control yogurt without BB-12, using the random allocation rule. Forty participants were recruited through Capital

Area Primary Care Research Network (CAPRICORN), a practice-based research network. The participants in the study were healthy individuals between the ages of 18-65 years and who were prescribed treatment with a penicillin class antibiotic regimen for an upper respiratory infection. A respiratory infection was classified as any infection the physician designates as strep or non-strep pharyngitis, otitis media, pneumonia, sinusitis or bronchitis that results in a 10-day prescription of antibiotics. All reported adverse events were tabulated by type and treatment group (see **Table 1**). There were no serious adverse events reported. No participants withdrew from the study due to adverse events.

Table 1. Phase I Adult Study Adverse Events

Event	Control Group (N=21)		BB-12 Group (N=19)	
	N	%	N	%
Abdominal pain	1	5	0	0
Acid reflux	0	0	3	16
Allergies (Seasonal, allergic rhinitis)	1	5	0	0
Back pain	0	0	1	5
Bloating	3	14	0	0
Bowel sounds	1	5	0	0
Breathing problems	3	14	0	0
Constipation	3	14	2	11
Cough	8	38	6	32
Decreased appetite	7	33	5	26
Diarrhea	2	10	2	11
Dizziness	0	0	1	5
Drug hypersensitivity	1	5	0	0
Ear aches	5	24	3	16
Fever	2	10	2	11
Gas	6	29	1	5
Headache	7	33	5	26
Irritability	1	5	1	5
Lethargy	8	38	4	21
Loose stool	9	43	4	21
Muscle pain	0	0	1	5
Nasal congestion	7	33	9	47
Nausea	1	5	0	0
Runny nose	7	33	5	26
Sore throat	5	24	6	32
Stomach pain	8	38	3	16
Tonsil swelling	1	5	0	0
Vaginal discomfort	1	5	0	0
Vomiting	0	0	1	5
Yeast infection	0	0	2	11
Total events reported	**98**		**67**	

II. Safety of BB-12 Supplemented Strawberry Yogurt For Healthy Children: The primary objective of our Phase 1 pediatric study was to assess the safety of strawberry flavored yogurt supplemented with *Bifidobacterium animalis* subsp. *lactis* strain BB-12 when consumed daily for 10 consecutive days by generally healthy children. The secondary objective was to assess the ability of BB-12 to survive gastric transit. The participants in the study were healthy individuals between the ages of 1-5 years. Participants were recruited through CAPRICORN. Twenty-nine participants received the BB-12 yogurt and 31 participants received the control yogurt. All reported adverse events were tabulated by type and

treatment group (**Table 2**). Three serious adverse events were reported (details in **Table 2**). No participant deaths were reported and no participants withdrew from the study due to adverse events.

Both studies, Safety of BB-12 Supplemented Strawberry Yogurt For Healthy Adults on Antibiotics and Safety of BB-12 Supplemented Strawberry Yogurt For Healthy Children were conducted under the same IND.

Table 2. Phase I Pediatric Study Adverse Events

	Days 0-10 (During Intervention)			Days 11-180 (Post-Intervention)		
Event	**Control**	**BB-12**	**Total**	**Control**	**BB-12**	**Total**
Allergies (e.g. seasonal)	1	2	3	1	3	4
Broken finger					1	1
Bronchiolitis					1	1
Cold	2		2	2	1	3
Constipation		3	3	2	1	3
Cough	5	10	15	1	5	6
Croup					1	1
Cut finger				1		1
Diarrhea	2	2	4	2	5	7
Ear aches					1	1
Ear infection				2	1	3
Fever		2	2	2	3	5
Flatulence	2	2	4	1	1	2
Headache				1		1
Hives		1	1			
Irritability	4	1	5	3	5	8
Laceration				1		1
Lack of/decreased appetite	2	2	4	3	1	4
Lethargy	1	5	6			
Loose stool	2	6	8	3	3	6
Lump on back of head				1		1
Nasal congestion	4	6	10		2	2
Pain	2	1	3	2		2
Physical injury				1		1
Pink eye					1	1
Pneumonia					1	1
Rash		2	2	1	2	3
Runny nose	6	12	18	6	9	15
Skin infection				1		1
Sore foot					1	1
Sore throat	1	2	3			
Strep throat					1	1
Umbilical hernia	1		1			
Vomiting		2	2	1	2	3
Total	**35**	**61**	**96**	**38**	**52**	**90**
Serious adverse events[a]		1	1		2	2

[a] Serious adverse events included: 1) grade 4/potentially life-threatening fever reported on day 2, 2) grade 4/potentially life-threatening bronchiolitis reported at day 180 and 3) grade 4/potentially life-threatening pneumonia reported at day 180. All serious adverse events were unrelated to the interventions and resolved.

***III. The Study to Investigate the Potential of Probiotics (SIPPY I and II)*:** We have also conducted two previous, non-IND but structure/function studies using the same investigational product, strawberry-flavored BB-12 supplemented yogurt. Both were double-blinded, randomized,

placebo-controlled, allocation concealment clinical trials consisting of a combined 354 healthy children between the ages of 1 and 5 years who attended daycare/school at least 3 days per week. The active BB-12 and control products were the same as used in the two Phase I trials. The primary objective was to determine if daily consumption of a probiotic-containing yogurt-based drink for 90 consecutive days decreased absences from daycare for the children, a structure/function outcome. Thus, the total days of BB-12 yogurt consumption was over 30,000 days. There were no significant differences in the days of missed school per group. Importantly there were no differences in adverse events (see **Table 3**) among BB-12 and control groups.

Table 3. SIPPY I and II Adverse Events

	Control	**BB-12**
SIPPY I Events	N=95	N=87
Number of subjects with at least one adverse event	3	3
Diarrhea	3	2
Pyrexia	0	1
Dermatitis (Diaper)	3	0
Vomiting & Cough (1 participant had 2 adverse events at the same time)	3	0
Hordeolum (Stye)	1	0
Number of subjects with at least one serious adverse event	0	0
SIPPY II Events	N=81	N=91
Number of subjects with at least one adverse event	3	8
Diarrhea	0	2
Hyperactivity	1	0
Rash	0	1
Frequent Stools	1	1
Stomach Pain	2	0
Constipation	0	1
Diaper Rash	0	1
Loose Stools	0	3
Number of subjects with at least one serious adverse event	0	0

2.11 Rationale

The rationale for focusing on food as a vehicle for the transmission of probiotics is that it has the potential to benefit a much larger public health population than using probiotics in a more medicinal manner, such as pills or capsules. Compliance with most medicinal regimens is around 50%; although certain interventions such as simplifying a regimen, collaboration with patients, different formulations, and increased convenience improve compliance.(99-102) We believe a readily available drink containing a high dose of probiotics has the potential to improve compliance through many of these mechanisms. This product also has the potential to positively impact the health of children and adults around the world, as yogurt will likely be more appealing to both children and their parents for long term consumption than pharmaceutical-like preparations. In addition to the benefits associated with the consumption of probiotics, there is an increased health benefit from consuming yogurt, a nutrient dense food.

2.12 Potential Risks and Benefits

2.12.1 Potential Risks

Yogurt: Potential risks and side effects related to the probiotic cultured yogurt include: allergic reaction to drink ingredients, most likely strawberry, or digestive problems such as stomachaches or loose, watery bowels due to lactose intolerance.

Amoxicillin-clavulanate: nausea and/or vomiting, diarrhea, upset stomach and mild skin rash. The most serious potential risk is diarrhea caused by a *Clostridium difficile* infection. Symptoms of *C. difficile* infection include stomach pain or tenderness, bloody stools, fever, nausea, and diarrhea.

There may also be side effects, other than those listed above that we cannot predict. Many side effects may go away shortly after the yogurt or antibiotic consumption is stopped, but in some cases side effects can be serious, long lasting or permanent.

Stool Collection: There is no known risk to stool collection. However, there may be some discomfort with collecting stool samples.

2.12.2 Known Potential Benefits

BB-12: *Bifidobacterium lactis* strain BB-12 is a commercially available probiotic strain used in a number of feeding and clinical trials. Such supplements have shown potential benefits in treatment and prevention of various diseases including diarrhea, asthma, necrotizing enterocolitis and allergies. It has also been found to significantly improve skin conditions in eczema patients.

Yogurt: Yogurt has its own known benefits coming from milk. For every 6-ounce serving, there are about 9 grams of animal protein, plus several other nutrients found in dairy foods, like calcium, vitamin B-2, B-12, potassium, and magnesium. Furthermore, yogurt with active cultures is found to help certain gastrointestinal conditions including, lactose intolerance, constipation, and diarrhea. Yogurt, which is more readily available and accepted by the general public, has the potential to greatly influence health if efficacious probiotic dosages and species are available in the product. Therefore, we believe that the benefits of the BB-12 supplemented yogurt far outweigh the discomfort and small risks participants might experience.

3. STUDY DESIGN

In order to determine the ability of BB-12 to impact antibiotic-induced perturbation and recovery, we will conduct a randomized, double-blinded, controlled trial in **60 healthy participants**, ages 18-65 years. The full inclusion and exclusion criteria are listed in Section 4.1. All participants will be given a 7-day prescription for 875/125 mg of amoxicillin-clavulanate (AMC), taken twice daily. In order to control for confounding by disease state and specific antibiotic used, all participants will receive the same daily dose --a four-ounce (approximately 100 ml) serving of probiotic (which delivers ≥10^{10} CFU of BB-12) or control yogurt-- and length of antibiotic treatment after a 30-day run-in period.

Participants are to provide informed consent on at Baseline, complete the 30-day run-in --during which they will refrain from all probiotics-- and begin the antibiotic and yogurt interventions on Day 1. As with our previous studies, enrollment and informed consent will occur at a private location, either in Dr. Merenstein's laboratory at Georgetown University Medical Center, or at the participant's home, as per the participant's preference. All interventions will be delivered in coolers to the participants' homes prior to day 1, at a time of their choosing.

Participants will be randomized to receive BB-12-supplemented yogurt and non-supplemented control yogurt (without BB-12), in a 2:1 (40:20 participants) allocation (**Figure 1**). Participants of all ages will consume the same dose of product per day for 14 days, at the same time as the AMC.

Fecal samples will be collected at home (twice at baseline (pre-run-in and post-run-in), and on days 7, 14, 21 and 30) into a maximum of three (1 sample for SCFA, 1 sample for microbiome testing, and 1 sample for potential future analysis) sterile, screw-capped tubes and frozen within one hour of collection in a home freezer. Participants will be given detailed, written and illustrated instructions on proper sample collection procedures, using protocols from the Fraser laboratory and as described in previous studies. Research staff will review these protocols with participants during enrollment. Immediately after collection and freezing, a member of the research team will be alerted to retrieve and transport the samples to the laboratory. The samples will be stored at -80°C until transport to The IGS. All staff will receive certification on handling procedures.

Follow-up phone visits will be completed on Days 7, 14, and 30. Participants will keep a daily diary to track number, shape and consistency of bowel movements, if/when antibiotic or yogurt was consumed, Bristol Stool chart, general health, use of other medicines or products, adverse events, and the GSRS - IBS instrument.

3.1 Primary Outcome Measure

The primary outcome is to determine the ability of BB-12 to impact antibiotic-induced reduction in SCFA as reflected by the levels of acetate on day 7, the most abundant primary colonic SCFA. SCFA produced by anaerobic gut microbiota will be quantified from human fecal samples via liquid chromatography-tandem mass spectrometry (LC-MS/MS).

3.2 Secondary Outcome Measures

The secondary aim is to determine the ability of BB-12 to impact antibiotic-induced disruption of the gut microbiota using 16S rDNA profiling. This will be assessed at days 7 and 14. Changes in absolute abundance of specific bacterial genera (with a particular emphasis on known butyrate producers in the Firmicutes phylum) will also be assessed with qPCR.

The experiments in the R61 phase of the study will provide sequential samples from which data on both microbial composition (based of 16S rDNA profiling) and total bacterial numbers (based on qPCR data) can be generated. Longitudinal 16S rDNA profiling will provide insight into several distinct shifts in the microbiota (e.g., overall decreases in bacterial numbers, loss of taxa, loss of overall community diversity) that reflect the impact of antibiotics (control group) and antibiotics plus BB-12 (active yogurt group). We will follow the extent and time course of recovery of the microbiota following cessation of antibiotic therapy.

We anticipate that a subset of participants, conservatively 15%, will develop diarrhea, and we will generate data on microbiota changes during the progression from the diarrheal state to the non-diarrheal state. This will allow us to ask if changes in the relative abundance of specific members of the gut microbiota (either alone or in combination) correlate with a clinical outcome.

The most likely outcomes from the study are:

1. +/- impact of BB-12 on fecal SCFA levels
2. +/- impact of BB-12 on gut microbiota composition (measured to quantify level of disruption achieved by antibiotics as a comparator within this study)
3. +/- impact of BB-12 on AAD (measured, but study not powered to detect clinical difference of AAD).

4. SELECTION AND ENROLLMENT OF PARTICIPANTS

4.1 Recruitment Strategy

We will enroll and randomize 60 participants; approximately 40 will be in the BB-12 group and 20 will be in the control yogurt. Statistical analysis on the primary outcome will be performed using the intention-to-treat principle; all subjects enrolled and randomized will be accounted for in the final analysis in their randomized group.

Flyers and posters will also be displayed in clinics, offices and community centers participating in the study prior to initiation.

The CAPRICORN research network is specifically designed to help conduct clinical trials. Ideas for studies either come from practicing physicians or University researchers. Regardless of where studies originate, they are then reviewed and sent out to physicians for comment. Thus, not all physicians or offices participate in each study; but are allowed to choose which studies that they have interest in and work within their office flow. Because of this grassroots approach, CAPRICORN has been very successful in fulfilling recruitment goals set forth a priori in studies. The network has helped the PI recruit over 1,500 participants in eight randomized controlled trials.

4.2 Inclusion Criteria

Participants will be *eligible* if:

1. S/he is between ages of 18-65 years
2. S/he has the ability to read, speak and write English
3. S/he has refrigerator for proper storage of the intervention
4. S/he has telephone access

4.3 Inclusion of Women and Minorities

This is an adult study and no one under age 18 will be included. All adults who meet the inclusion/exclusion criteria, regardless of religion, sex, or ethnic background will be enrolled if they sign the informed consent.

Based on rates of patients seen in our network, we anticipate about 50% of the potential participants will be female. Many of our practices are based in the inner-city and have an over-representation of ethnic minorities.

4.4 Exclusion Criteria

Participants will be *ineligible* if any of the following conditions are present:

1. Diabetes or asthma that requires daily medication
2. Allergy to strawberry
3. Active diarrhea (three or more loose stools per day for two consecutive days)
4. Any gastrointestinal medications, i.e. medicines for irritable bowel syndrome, gastroesophageal reflux disease, inflammatory bowel disease, etc. (a full medication list will be reviewed by the PI prior to enrollment)
5. Lactose intolerance
6. History of heart disease, including valvulopathies or cardiac surgery, any implantable device or prosthetic
7. History of gastrointestinal surgery or disease
8. Milk-protein allergy

9. Allergy to any component of the product or the yogurt vehicle
10. Allergy to penicillin or cephalosporin class antibiotics
11. Allergy to any of the following medications:
    a. Penicillin
    b. Erythromycin
    c. Tetracycline
    d. Trimethoprim
    e. Ciprofloxacin
12. Women who are breastfeeding, pregnant, or planning to become pregnant during the study.

Participants must agree to refrain from all probiotics throughout the study, starting from the 30-day run-in period through day 30.

4.5 Study Enrollment Procedures

Research staff will meet with area physicians via the CAPRICORN network and community members to inform them about the study. Flyers and posters will also be displayed in clinics, offices, and community centers participating in the study approximately three months prior to initiation of the study. Information about the study procedures and criteria will be available on a public, study-specific website (content pre-approved by the GU IRB).

Potential participants will be pre-screened over the phone or in-person using the Pre-screening Form (PS). If they meet basic entry criteria, the potential participant will be offered the opportunity for study participation and will be given a copy of the informed consent form and/or link to the website, and time to review before making the choice about participation. If the participant agrees, an enrollment and informed consent visit will be scheduled at a mutually agreed time and location. Information provided during the consent process include, the purpose of the study, procedures, withdrawal procedures, subject termination, risk/discomforts, benefits, costs, compensation, and alternatives to participation.

This study design is such that potential participants sign the informed consent about 45-60 days before starting any interventions. About 1-3 weeks after signing the informed consent, the participants have at least a 30-day wash out period where they refrain from consuming any probiotic products and antibiotics; after which, they will start the intervention period. Due to the time lag between enrollment and the start of the interventions, some participants decide to withdraw in the interim before starting any interventions. Since this is a study to understand the mechanisms of preventing antibiotic-associated diarrhea using probiotics, in order to accomplish the study aims, it is critical to have data collected from 60 participants who have been randomized and commenced the interventions. As such, we will enroll approximately 100 individuals to account for the withdrawal rates, so that study is completed with a final sample size of 60 participants.

5. STUDY INTERVENTIONS

5.1 Interventions, Administration, and Duration

The probiotic-supplemented yogurt is composed of the investigational agent, BB-12, and contains the yogurt starter culture YF-L702 (a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*). The yogurt starter culture will be present and alive in the control yogurt. Starter culture alone is unlikely to impact AAD, as it does not survive the acidic stomach environment and is not recovered alive in the feces. Details about yogurt preparation, and enumeration and PCR verification of BB-12 have been previously published and reviewed by the United States Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER).

All participants will take the same amount of either the BB-12 or control yogurt, as determined by random assignment. Participants will each consume a four-ounce daily dose orally for 14 days while on amoxicillin-clavulanate for 7 days. Each four-ounce daily dose of the active yogurt will deliver no less than 10^{10} CFU of BB-12 per day.

Study interventions	BB-12 yogurt and amoxicillin-clavulanate, or Control yogurt and amoxicillin-clavulanate
Administration	Oral, to individual participants
Dosing schedule	4-ounces daily of BB-12 or control yogurt on days 1-14, and Amoxicillin-clavulanate 875/125 mg twice per day on days 1-7
Possible adverse events	See Section 7.3
Setting	Community
Intervention delivery	Research personnel will provide interventions Participants will self-administer
Use of appropriate supportive care, medications or treatments	Not applicable
Dose escalation procedures	Not applicable
Instructions for modifications	Not applicable
Package insert information	See package insert for amoxicillin-clavulanate

5.2 Handling of Investigational Product

5.2.1 Study Product Description

5.2.1.1 Acquisition

The investigational agent, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strain BB-12 (BB-12), will be supplied by manufacturer, Chr. Hansen, in Milwaukee, WI.

Chr. Hansen, Inc.
9015 West Maple Street
Milwaukee, WI 53214

The investigational agent will be shipped directly from the manufacturer to The Pennsylvania State University where they will be added to the yogurts.

The Pennsylvania State University
Department of Food Science
428 Food Science Building
University Park, PA 16802

5.2.1.2 Formulation, Packaging, and Labeling

The probiotic-supplemented yogurt is composed of the investigational agent, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strain BB-12 (BB-12), and also contains the yogurt starter culture, YF-L702 (a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*).

The Chr. Hansen Culture Collection (CHHC) numbers of the exact strains used in the manufacture of the yogurt blend YF-L702 are *Streptococcus thermophilus* ST5389, *Streptococcus thermophilus* ST5086, *Streptococcus thermophilus* ST4460, *Lactobacillus delbrueckii* subsp. *bulgaricus* LB4351, and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB2164.

Once manufactured, the probiotic-supplemented yogurt-drink will be packaged in pint-size plastic containers, which will yield 4 servings of the 4-ounce daily dosage. Four containers of the yogurt drink with their randomly assigned bin number will be provided to the participants after enrollment and the 4-week run-in period, prior to day 1, along with the plastic cups and markings that denote levels of 2, 4, 6, and 8 ounces. The daily dose is one serving of 4-ounces of yogurt each day. This will deliver no less than 10^{10} CFU per day of BB-12.

Each plastic container will bear the label “Caution: New Drug-Limited by Federal (or United States) law to investigational use” in addition to its manufactured date and time.

5.2.1.3 Yogurt Drink Manufacture

Yogurt drink manufacture is broken into two major activities, 1) preparation of the yogurt and 2) blending with other ingredients to prepare the drink. To make the yogurt, a yogurt base is formulated to contain 9.0% milk solids non-fat (MSNF), 1.3% fat and 3.0% sugar using skim milk, cream, non-fat dry milk and sugar. The wet ingredients (skim milk and cream) are mixed together and then blended with previously weighed dry ingredients (sugar and non-fat dried milk). After blending the yogurt mix is pasteurized and homogenized (2000 psi first stage, 500 psi second stage) at 83°C for 29 sec. The pasteurized, homogenized mix is pumped to fermentation tank and given an additional heat treatment of 85°C for 30 min to denature whey proteins, then cooled to 42°C prior to inoculation with 0.02% of the yogurt starter culture (YFL-720). The inoculated mix is agitated for 15 minutes and then allowed to incubate quiescently until the pH reaches 4.6.

To prepare the yogurt drink the gel structure of the fermented yogurt is disrupted by agitation and blended with a mixture of pectin, 36 DE corn syrup solids, sugar and water. The pectin-containing mixture is prepared by heating water, sugar, 36DE corn syrup solids, and pectin to 80°C, holding for 1 hour. This mixture is then cooled to 40°C and added to the fermented white mass resulting in the unflavored yogurt drink base. To flavor the product, strawberry puree is then added to the yogurt drink. The composition of the yogurt drink is shown in Table 4 and the ingredients used in manufacture are listed in Table 5. At this point, a portion of the yogurt drink is homogenized (1500 psi first stage, 500 psi second stage) for a second time to create a drinkable consistency and packaged in plastic bottles. To prepare the yogurt drink containing BB12, an appropriate amount of (BB-12) is added to the yogurt drink, allowed to mix for 15 minutes and then homogenized and packaged as described above. The packaged yogurt is stored at 4°C.

Table 4. Composition of Strawberry Yogurt Drink

Component	Composition in Yogurt Drink
Fat	1.0%
Milk Solids Non-Fat	6.8%
Sucrose	6.1%
Corn Syrup Solids	6.4%
Pectin	0.4%
Strawberry Puree	3.0%

Table 5. Ingredients for Strawberry Yogurt Drink

Component	Supplier	Specifications
Milk[1]	Land-O-Lakes	PMO[2]
Nonfat Dry MilkSolids[3]	Various approved vendors	COA[4]
Granulated Sugar[3]	Various approved vendors	COA
Corn Syrup Solids[3] (42 DE)	Various approved vendors	COA
Pectin ADM 783	DuPont, New Century, KS	COA
Strawberry Puree #242529384	Sensient Flavors, Amboy, IL	COA
YF-L702 (starter culture)	Chr. Hansen, Milwaukee, WI	COA
BB-12 (probiotic)	Chr. Hansen, Milwaukee, WI	COA

[1] Milk is received on an as needed basis and is purchased from Land-O-Lakes.
[2] Milk will be produced under and meet the requirements of the Pasteurized Milk Ordinance (PMO).
[3] The supplier for nonfat dry milk, sucrose and corn syrup solids may change based on availability and price of ingredient.
[4] All ingredients will be food-grade and a will require a Certificate of Analysis (COA) from the supplier.

5.2.1.4 Enumeration of BB-12 from the Yogurt

The viable count of BB-12 in the product is measured the day of manufacture (Day 0) and weekly until Day 30 of shelf-life. The yogurt drink is diluted in sterile peptone water blanks (3M, St. Paul, MN) and then appropriate dilutions of the product are cultured by pour plating on an MRS-based selective bifidobacteria medium.(103) Modified MRS agar is made by adding 4.5 grams Agar (Difco, BD, Sparks, MD) to 300 ml MRS broth (Difco, BD, Sparks, MD). After autoclaving, the agar is used immediately after addition of three selective components, 1.5mL of 0.01% Dicloxacillin (Sigma-Aldrich, St. Louis, MO); 3.0 mL of 10% Lithium Choride (Sigma-Aldrich, St. Louis, MO); and 1.5 mL of 10% Cysteine Hydrochloride (Sigma-Aldrich, St. Louis, MO). Plates are anaerobically incubated (VWR, West Chester, PA) at 37°C and counted after 72 hours. The final yogurt drink also is evaluated for the presence of coliforms using high sensitivity PetrifilmTM Coliform Count Plates (3M™, Burlington, North Carolina) and Petrifilm™ aerobic plate counts according to the 17th edition of Standard Methods for the Examination of Dairy Products, #7.072 and #6.040, respectively.

5.2.1.5 PCR Verification of BB-12 Level in Finished Yogurt

To further verify the selectivity of the BB-12 agar, colonies of varying morphology are selected for identification using *B. lactis* specific PCR primers according to the method of Ventura et al.(104) Cells are lysed according to the microwave method of Bollet et al. as modified by Kullen et al.(105, 106) The sequence of the forward primer, Bflact2, is 5'-GTGGAGACACGGTTTCCC-3' and the reverse primer, Bflact5, is 5'-CACACCACACAATCCAATAC-3'. Amplicons from PCR are electrophoresed through a 2% agarose gel at 110 volts for 2 hours, stained with ethidium bromide (Promega, Madison, WI), destained with distilled water and visualized using an Alpha-Imager (Alpha Innotech, San Leandro, CA). The presence of amplicons of the appropriate size is taken as evidence the colonies are indeed *B. lactis*.

5.2.1.6 Acceptability Testing

We also completed two previous studies using the same BB-12 yogurt drink in a combined total of 354 children ages 1-4 years to determine the effects of BB-12 yogurt on daycare absences. Participants consumed the same amount of yogurt we are proposing for this trial (four ounces daily) for 90 consecutive days. Only minimal adverse events were reported by 17 participants in over 30,000 total days of product consumption; all were non-serious and self-limited. High rates of compliance, over 92%, were reported in both trials, indicating the safety and tolerability of the BB-12 yogurt drink, at this dosage.(107, 108)

5.2.1.7 Final Product

Table 6 contains information about the initial population of BB-12 in the product as well as the results of viable count analysis conducted up through the end of shelf life for 42 separate yogurt-drink manufacturing experiments. The total solids of the product ranged from 20.53 to 22.11% with a mean of 21.2%. The fat content of the product ranged from 0.52 to 1.29% with a mean of 0.9%. The pH of the product ranged from 4.42-4.7 with a mean of 4.42 on the day of manufacture and remained relatively constant throughout shelf life. The population of BB-12 declined slowly throughout the self-life but remained within the target level (log 9 CFU/g). Viable counts of BB-12 remained about the target value in subsequent weeks (data not shown) but we chose to set the shelf life at 30 days. **Thus a 100 ml serving would supply no less than 10^{10} CFU per day of BB-12.**

Table 6. pH and BB-12 Values for Yogurt Drink During Storage

Time	pH (± SD)	Log CFU/ml of BB 12 (± SD)
Day 0	4.41 (0.13)	8.86 (0.36)
Week 1	4.42 (0.12)	8.86 (0.32)
Week 2	4.39 (0.11)	8.65 (0.39)
Week 3	4.38 (0.12)	8.48 (0.47)
Week 4	4.42 (0.12)	8.49 (0.40)

The release criteria for the product will be similar to fresh dairy products. The proposed release criteria are shown in **Table 7**.

Table 7. Release Criteria for finished product.

Item	**Acceptable range**	**Method**
Total Solids	20.5-22.5%	Microwave Drying[1]
Total Fat	0.5-1.5%	NMR[1]
pH	4.1-4.7	pH Meter
Coliforms	Negative by test	Method #7.072[2]

[1]Analysis obtained using CEM SMART Trac Fat and Moisture Analyzer, Matthews, NC
[2]17th Edition of Standard Methods for the Examination of Dairy Products

A mock nutrition facts panel for the yogurt drink is shown in **Figure 1**, as well as a sample of the label, which is placed on the final product when used as food (**Figure 2)**.

Figure 1. Mock nutrition label for probiotic strawberry yogurt.

Nutrition Facts Serving Size: 100g	
Amount Per Serving	
Calories 110	Calories from Fat 10
	% Daily Value*
Total Fat 1.5g	2%
Saturated Fat 1g	5%
Trans Fat 0g	
Cholesterol 5mg	2%
Sodium 55g	2%
Total Carbohydrate 23g	8%
Dietary Fiber 0g	0%
Sugars 19g	
Protein 3g	
Vitamin A 0% •	Vitamin C 2%
Calcium 10% •	Iron 0%
*Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:	

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g
Calories per gram: Fat 9 ● Carbohydrate 4 ● Protein 4			

Ingredients: Cultured pasteurized skim milk, sugar, corn sweeteners, strawberries, cream, non-fat milk solids, pectin, modified food starch (corn), natural flavors, sodium citrate, potassium sorbate (preservative), red 40, blue 1.

Figure 2. Mock Product Label

5.2.2 Dosage, Preparation and Administration of Investigational Product

Enrolled participants will be given a total 14-day supply of the yogurt following the 4-week washout period. Participants will drink four (4) ounces of yogurt each day for 14 consecutive days. Yogurt will be administered via mouth.

5.2.3 Accountability Procedures for the Investigational Product

Written records of receipt and storage of the investigational product, including date of receipt, quantity received, amount distributed to participants, and final disposition will be maintained. Any known discrepancies in the accountability will be documented. The investigator will not use the product in an investigational manner other than that provided for in the protocol; however the product is also a yogurt and the investigator requests the use of the surplus yogurt for teaching, charitable, and other educational purposes.

5.2.4 Product Transportation, Handling and Storage

5.2.4.1 Transportation and Handling

The BB-12 and control products will be transported under refrigeration in coolers packed with ice from the Penn State area via a van driven by Penn State personnel. A member of the study personnel will meet the Penn State van at a designated meeting point halfway between the two campuses. The Study personnel will then transport the products, still in its coolers with ice, the remainder of the trip to the Washington, DC area. While in transport, each cooler will be equipped with VWR® Digital Refrigerator/Freezer Thermometer with Alarm, which will alert the study personnel by audible alarm if the

temperature of the coolers increased above the preset acceptable levels (between 32°F and 45°F) at any time during transport.

5.2.4.2 Storage After Arriving in Washington, DC Area

Once the yogurt drinks arrive at Georgetown University in Washington, DC, they will immediately be transferred to two refrigerators that are exclusively used for the study. Each refrigerator is equipped with a lock and a thermometer. Study personnel will take a daily temperature reading in each refrigerator to ensure that the yogurt drinks are kept between 32°F and 45°F. Each refrigerator is equipped with a SENSAPHONE® 2.8K Weatherproof Temperature Sensor, which are connected to a SENSAPHONE® 400 Remote Monitoring and Alarm Notification System. The sensor system will trigger an alarm when the temperature falls below or rises above customizable limits (between 32°F and 45°F) or if there is a power failure, and will contact up to four study personnel phone numbers.

BB-12 and control yogurt products expire one month from the date of manufacture. The date of manufacture can be found printed on each bottle of the yogurt drink. Approximately every three weeks, study personnel will make arrangements with Georgetown University Facilities Management to dispose of the products before or on the date of expiration.

5.2.4.3 Transportation and Storage While Being Dispensed to Participants

The products will be transported to the participants' homes in cooler bags. Prior to each delivery, the study personnel will arrange and confirm a mutually convenient time with the participants to ensure that they will be home to receive the drink. This ensures that the participants can place the yogurt drinks into their home refrigerators immediately upon receipt.

5.2.4.4 Duration of Storage

The products are stored in the study refrigerators at Georgetown University from the day they are delivered from Penn State (same day delivery) until transported via coolers to study participants. As mentioned above, all bottles are labeled with the dates of manufacture and will thus be stored at Georgetown University until expiration—one month after the manufacture date. Extra yogurt may be delivered upon request by the participants due to spillage, spoilage or any other reasons as needed.

5.2.4.5 Instructions to Participants Regarding Storage and Dosing

BB-12 and control yogurt products expire one month from the date of manufacture. The date of manufacture can be found printed on each bottle of yogurt drink. Since the drinks are only to be consumed during a 14-day period and deliveries to participants will coincide with the arrival of fresh product from Penn State, participants should always have enough unexpired product in their possession to continue the study. Participants will also be advised to dispose of any product that has been left out of the refrigerator for longer than one hour. In such cases, they may contact study personnel for fresh yogurt drinks to be delivered.

5.2.5 Assessment of Subject Compliance with Investigational Product

DNA extracted from stool samples collected at baseline and at day 7 will be amplified using *B. lactis* specific PCR primers, in order to assess compliance. Participants will not be withdrawn from the study for missed specimen collections but it will be noted in the study file.

5.3 Concomitant Interventions

5.3.1 Allowed Interventions

Once enrolled, participants are allowed interventions or medications as needed, at the guidance of their

personal physician. Since the use of additional or concomitant interventions could bias results, participants will be asked to report any of these additional treatments. Participants will be advised multiple times before enrollment to avoid concomitants, but once enrolled they will be told of the importance of articulating usage. All participants will be given a list of products they should avoid during the study. Due to the intention-to-treat principle, participants will not be removed from the study but will continue with all planned study interventions and data collection.

If the participant takes any antibiotics within the 30-day run-in period, they will repeat another 30-day run-in upon completion of the antibiotics, prior to initiation of the interventions.

5.3.2 Required Interventions

Probiotic supplemented yogurt or control yogurt, and amoxicillin-clavulanate provided by the study.

5.3.3 Prohibited Interventions

Participants will be *ineligible* if they are taking the following medications at the time of enrollment:

- Daily diabetes or asthma medication
- Any gastrointestinal medications, i.e. medicines for irritable bowel syndrome, gastroesophageal reflux disease, inflammatory bowel disease, etc. (a full medication list will be reviewed by the PI prior to enrollment).

Participants must also avoid the following for the duration of the study:

- Any probiotic not provided by the study.
- Any antibiotic not provided by the study.

5.4 Adherence Assessment

We will assess adherence to the yogurt interventions in four distinct manners. First, we will assess adherence to the antibiotic and yogurt interventions via the daily assessment diary. Second, participants will take photos of the remaining antibiotics and yogurts bottles at the end of the intervention periods and send them to the research assistant. Third, at all follow-up phone calls, participants will be asked if they consumed the yogurt each day and how much was consumed.

Protocol compliance is defined as consuming 2 or more ounces of the assigned study yogurt per day, for at least 11 of the 14 days. Participants will not be withdrawn from the study for missed interventions or specimen collections, but these adherence assessments may be used for any per-protocol analyses.

The fourth measure of adherence will be DNA extracted from fecal samples collected at baseline and at day 7. This DNA will be amplified using *B. lactis* specific PCR primers to assess compliance with the yogurt interventions. Fecal samples are thawed on ice to remove a representative 1 gm sample for DNA extraction using the QiaAmp DNA Stool Mini Kit (Qiagen Sciences, Valencia, CA). The Kit's procedure is followed using the additional 5 minute heating step at 95°C. The isolated DNA samples are subjected to *B. lactis* specific PCR analysis. To further verify the selectivity of the BB-12 agar, colonies of varying morphology are selected for identification using *B. lactis* specific PCR primers according to the method of Ventura et al.(104) Cells are lysed according to the microwave method of Bollet et al. as modified by Kullen et al.(105, 106) The sequence of the forward primer, Bflact2, is 5'-GTGGAGACACGGTTTCCC-3' and the reverse primer, Bflact5, is 5'-CACACCACACAATCCAATAC-3'. Amplicons from PCR are electrophoresed through a 2% agarose gel at 110 volts for 2 hours, stained with ethidium bromide (Promega, Madison, WI), destained with distilled water, and visualized using an Alpha-Imager (Alpha Innotech, San Leandro, CA). The presence of amplicons of the appropriate size is taken is taken as evidence the colonies are indeed *B. lactis*.

6. STUDY PROCEDURES

6.1 Schedule of Evaluations

All interventions will be completed by day 14 of the trial. At day 30, participants will provide the final sample and be queried for changes in baseline health and other adverse events.

Table 8. Participant Timeline and Sample/Data Collection Schedule (N=60)

Assessment	**Pre-screen**	**Pre Run-In** Baseline	30-day Run-In	**Post Run-In** (0/+2)	**1**	**7** (±2)	**14** (±2)	**21** (±2)	**30** (±2)
Pre-Screening	X								
Informed Consent Form & Process		X							
Inclusion/Exclusion Criteria		X		X					
Enrollment		X[a]							
Baseline Health Status		X							
Demographic Information		X							
Fecal Microbiome Collection[a]		X		X		X	X	X	X
Automated Self-Administered 24-Hour (ASA24®) Dietary Assessment Tool (2 days)			X						
Run-in Period (30 days)			|→	→|					
Post Run-In Health Status				X					
Randomization				X					
Amoxicillin-clavulanate Intervention (7 days)					|→	→|			
Yogurt Intervention (14 days)					|→	→	→|		
Follow-up Data Collection						X	X		X
Daily Assessment Diary[b] (14 days)					|→	→	→|		
Gastrointestinal Symptom Rating Scale (GSRS-IBS)					X	X	X		
SCFA Testing		X				X			X
16S rRNA Gene Sequences				X		X	X	X	X
Adverse Event Reporting		|→		→	→	→	→	→	→|

[a]Enrollment will occur a minimum of 30 days prior to starting the interventions. Participants will provide one stool sample before the run-in period, refrain from antibiotics and probiotics during this period and provide 1 baseline sample after the run-in period.
[b]Diary from days 1-14; information on yogurt consumption/dose, sample number, shape and consistency, health, use of interventions, quality of life and adverse events.
X: collected on day; |→: starting from day; →|: through end day

6.2 Description of Evaluations

All laboratory and clinical measurements, as well as method of data collection, are listed in **Table 9**.

Table 9. R61 Phase Assessment	**Collection Method**
Impact of antibiotics on gut microbiota	Bacterial number: real-time qPCR; Overall microbiota composition: 16S rDNA profiling
Impact of BB-12 on gut microbiota	Bacterial number: real-time qPCR; Overall microbiota composition: 16S rDNA profiling
Impact of BB-12 on diarrhea/stool frequency	Diary - patient reported outcome
Impact of antibiotics on metabolism of gut microbiota	Fecal SCFA quantification
Impact of BB-12 on metabolism of gut microbiota	Fecal SCFA quantification
Bristol Stool Chart	Diary - patient reported outcome
Gastrointestinal Symptom Rating Scale - IBS version (GSRS-IBS)	Diary - patient reported outcome
Compliance for antibiotics and probiotic interventions	DNA extracted from fecal samples; daily diary; photo documentation of intervention bottles; follow-up visits

6.2.1 Sample Collection and Analyses

6.2.1.1 Sample Collection

Stool samples will be collected into three sterile, screw-capped tubes and frozen immediately. The participants will be instructed on how to collect the samples and be provided with written, illustrated instructions. (Refer to Section 6.2.3, “Stool Collection Instructions for Participants.”)

6.2.1.2 Transport

After collection, a member of the research team will be phoned and instructed to retrieve the samples and transport them to the laboratory. As part of their training, students and the entire research team will sign an informational sheet documenting their understanding of the risk of handling potentially infectious materials. The stool will be transported back to the lab and placed in -80^0 Celsius to freeze for batch transport to the Institute for Genomic Sciences at the University of Maryland Baltimore (Fraser Lab). (Refer to Section 6.2.2, “Stool Collection Instructions for Study Personnel.”)

6.2.1.3 Short Chain Fatty Acid (SCFA) Analysis

Primary fecal SCFA produced by anaerobic gut microbiota in the colon (acetate, propionate, butyrate) will be quantified via a liquid chromatography-tandem mass spectrometry assay. Primary fecal SCFA produced by anaerobic gut microbiota in the colon (acetate, propionate, butyrate) will be quantified via a liquid chromatography-tandem mass spectrometry assay. Fecal samples will be homogenized in 50% aqueous acetonitrile, vortex mixed and centrifuged to yield a supernatant containing SCFAs. SCFA in the supernatant are then derivatized with 3-nitrophenylhydrazine to enhance sensitivity of detection. Heavy isotope labeled derivatizing agent ($^{13}C_6$-3nitrophenylhydrazine) is reacted with authentic standards of SCFAs to generate stable isotope-labeled internal standards for each analyte. SCFA will be separated using a Waters BEH C18 (2.1 x 100mm, 1.7micron) UPLC column using an acetonitrile/water/formic acid-based gradient mobile phase where LC-MS/MS analysis will be conducted on either an AB Sciex 5500 QTRAP or a Thermo TSQ QuantumUltra triple quadrupole mass spectrometer operated in negative ion mode.

6.2.1.4 Microbiome Community Profiling

The gut microbiota will be characterized by sequencing bacterial 16S rDNA gene amplicons from fecal samples as previously described.(109-113) Raw 16S rDNA reads will be processed using published protocols.(110-112) Within- (α-diversity) and between- (β-diversity) sample comparisons will then be performed using QIIME.(113) Microbiota will be compared cross-sectionally and longitudinally, with emphasis on parameters such as the Bray-Curtis similarity score,(114) Chao1 metric (α-diversity or richness), the Shannon index (abundance and evenness of species in a community), determined using standard tools, such as Mothur,(115) QIIME,(113) and statistical R package (www.r-project.org). In addition, statistically significant correlations between specific microbiota members and clinical outcomes will be determined using bioinformatics tools, such as eLSA,(116) Metastats,(117) and LEfSe.(118) The 16S rRNA dataset will also be used to simulate the function of the microbiota using PiCRUSt(119) which uses available genome sequence data to infer functional gene contents based on the 16S rRNA microbiota composition.

6.2.1.5 Real Time qPCR Analysis of Total Bacterial Number

Reactions will be performed in duplicate using the microbial qPCR DNA kit (Qiagen) and analyzed on the

Fluidigm Biomark platform.

6.2.2 Stool Collection Instructions for Study Personnel

- Inform the participants of the following:
  1. Refer to “Stool Collection Instructions for Participants” for detailed instructions.
  2. Use the kit provided to collect sample.
  3. All stool samples must be frozen immediately.
  4. Make sure to use gloves when handling sample.
  5. Write the collection time and date on the label provided.
  6. Call the study personnel after collecting the sample to schedule a pick up.
- Use the cooler/coolly packs when transporting samples between the participant’s house to Georgetown University.
- Confirm participant or a housemate will be available in the time window.
- Upon receipt of the sample, check the label; if no date/time was marked on the sample, ask the participant to complete.
- Fill out **Sample Collection (SC) Form,** *even if the sample was not collected* (check “no” on form).
- Before placing the sample in the freezer, make sure:
  1. Any names on the samples are crossed out
  2. Write correct Study IDs
  3. Make sure all vial caps are tightly closed.

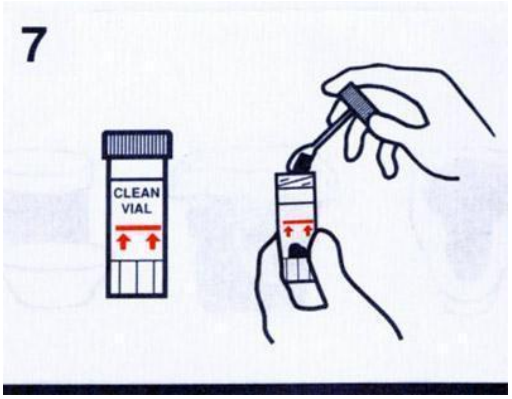
6.2.3 Stool Collection Instructions for Participants

Stool specimens are to be collected 6 times during the study: before the run-in period, after the run-in period, and on days 7, 14, 21 and 30. At your enrollment visit and throughout the study, you will be provided with stool collection materials. Each kit will include: disposable gloves, 3 stool vials/containers, small plastic zipper bags, 1 large plastic zipper bag, labels and a stool collection device (“stool hat”).

Before you begin the collection, please clear a space or shelf in your freezer to store the samples without contact with food or other items in the freezer. At the time of stool collection:

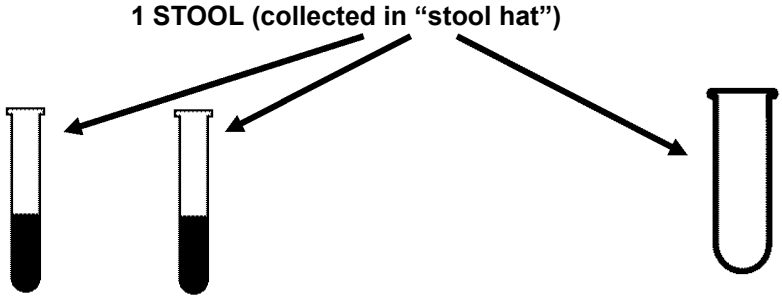
1. Wash your hands.
2. Put on disposable gloves.
3. Each kit will include labels with your **STUDY ID** number and the sample date (pre-run-in, post-run-in, day 7, day 14, day 21 or day 30). If it is not already on the label, please write in your **STUDY ID** number, as well as the **DATE** and **TIME** the stool was collected. Please label each of the 3 stool vials and once on the label on the plastic zipper bag. **Please do not write your name on the labels**.
4. Lift the toilet seat. Align the “stool hat” with the rear of the toilet rim (see **Photo A**). Replace the seat. Use the toilet as you normally would, and let the stool collect in the “hat”.
5. Unscrew the cap from one of the smaller vials that contains liquid, without spilling any of the liquid inside. Using the spoon attached at the cap of the vial, fill the vial to the “fill line” indicated on the vial (but do no overfill; see **Photo B**).

Photo A. 

Photo B. 

6. Screw the cap back onto the vial. Make sure the cap is tightly closed. Shake the vial gently until the stool is thoroughly mixed with the liquid. **REPEAT** the same action for the second smaller vial with the liquid (see **Figure 1)**.
7. For the last vial (the larger vial without liquid), simply collect stool to the fill line on the vial using the attached spoon and screw the cap back onto the vial (see **Figure 1**).

Figure 1.



3 VIALS: VIAL 1 (LIQUID) VIAL 2 (LIQUID) VIAL 3 (EMPTY)

Please avoid contact with the liquid preservative in the stool vials.
If any of the liquid comes into contact with skin or eyes, wash thoroughly with water. Do not ingest/drink the preservative.

8. Place the vials into the small plastic zipper bag and seal the bag. Then place the small bag into the large plastic zipper bag. Seal the bag.
9. Discard remaining stool into the toilet and flush. Discard stool hat.
10. Place the sealed plastic zipper bag into the **FREEZER immediately** for storage. Avoid contact with food or other items in the freezer.
11. Discard gloves into the trash and wash your hands.
12. Contact the research staff as soon as possible to arrange a time for stool collection pickup, which should typically occur within 48 hours.

6.3 Participant Stipends

Participants will receive up to $120 total in compensation for their time. $20 will be paid for each stool specimen collected for a total of six specimens. . All fecal sample tests will be paid for by the study and participant's insurance company will not be responsible for tests. As per Georgetown University IRB protocol and will be elucidated in the Informed Consent, the PI, Georgetown University, Penn State

University and no parties involved in the study will be responsible for additional medical care that participants may need.

6.4 Screening Evaluation and Consenting Procedure

Individuals who are interested in participating in this study will contact a member of the research team by phone, e-mail, or in-person. A member of the research team will establish contact with the individual, either by phone or in-person, and provide a brief overview of the study and answer any questions. If the individual would still like to participate, the research coordinator or assistant will ask questions from a pre-screening form to assess eligibility and exclusion criteria. A visit for enrollment and informed consent will be arranged at the participant's home or the Merenstein lab at the Georgetown University Medical Center, per the participant's preference.

If the basic screening criteria are met, the potential participant will be offered the opportunity for study participation and consent will be obtained in-person. Information provided during the consent process include the purpose of the study, procedures, withdrawal procedures, study termination, risk/discomforts, benefits, costs, compensation, and alternatives to participation.

If the individual does not qualify after the pre-screening, or decides not to participate, the pre-screening form will document the reason, and will be filed separately from the participant records, without personally identifiable information. The research staff will maintain a screening log to track screen fails and enrollments.

A single informed consent form is initiated prior to an individual's agreeing to participate in the study and continuing throughout an individual's study participation. Extensive discussion of risks and possible benefits of this study will be provided to the participants. A single consent form describing in detail the study interventions/products, study procedures, and risks, are given to the participants and written documentation of informed consent is required prior to starting intervention/administering study product. The research team will discuss all information outlined in the informed consent document with prospective participants to ensure that they understand the nature of the research and can voluntarily decide whether or not to participate.

Research staff will document the following on the Informed Consent Process Documentation form: a) Were the Informed Consent Form, Health Insurance Portability and Accountability Act (HIPAA) Authorization and related study documents thoroughly reviewed with the study participant?; b) Did the study participant have sufficient time to review the documents and ask questions?; c) Was the Informed Consent and HIPAA Authorization signed prior to the initiation of any study related procedures?; d) Were copies of the signed documents given to the study participant?

6.5 Enrollment, Baseline, and Randomization

6.5.1 Enrollment

The enrollment/baseline visit will take place at the participants' homes or an office where comfort and confidentiality is assured. There is no clinic visit associated with this study. The purpose of this visit is to have in-person time to collect baseline data, explain all forms, go over the schedule of visits and intervention, and give the participant the initial compensation. No physical exams or medical assessments will be conducted as part of the screening or enrollment process.

6.5.2 Baseline Assessments

Once informed consent is given and it is confirmed that the participant meets all inclusion and exclusion criteria, baseline data will be collected. These include: Baseline Health Status and Demographic Information.

Stools will be collected multiple times throughout the study. Research staff will be trained on how to properly transport stool and how to instruct participants on collecting stool.

During the initial visit, participants will also be given all materials to collect stools, the diary, and study materials as well as the initial compensation. The participant will commence the 30-day run-in period. During this 30-day run-in period, the participant will refrain from antibiotics and probiotics (a “What Not to Eat list will be provided) and provide two baseline stool samples--one pre run-in, and one post run-in (prior to starting the yogurt and antibiotics).

Additionally, the research staff will phone the participant two times during the run-in period to collect 24-hour diet recall data. The instrument, the Automated Self-Administered 24-Hour (ASA24®) Dietary Assessment Tool, will be administered by the research staff and recorded for one weekday and one weekend day (https://epi.grants.cancer.gov/asa24/.

Following the run-in period and prior to the yogurt start day (day 1), research staff will contact the participant in order to complete the Post Run-in Health Status form and repeat the Inclusion/Exclusion form to ensure that the participants are still eligible. Participants who start an exclusionary medication or experience an onset of an exclusionary, nontransient health condition from the time of enrollment will be withdrawn from the study.

Should the participant take any antibiotics during this 30-day run-in period, they will start another 30-day run-in cycle after the antibiotics are completed. The initiation of the yogurt and antibiotic interventions (day 1) will be delayed until this additional run-in cycle is complete. Participants who take any non-study probiotics during this 30-day run-in period will document any use on the Post Run-In Health Status form. Participants who need to delay their day 1 (initiation of the yogurt and antibiotic intervention), e.g. to complete a full 30-day run-in or due to transient conditions, can be held for a future cohort for up to 90 days from the day the informed consent was signed, without the need to re-consent. After 90 days, the participant will be re-consented prior to starting the interventions.

Participants who are still eligible based on the completion of the Post Run-in Health Status and post run-in Inclusion/Exclusion forms will then be randomized to receive the BB-12 yogurt or control yogurt for days 1 through 14 to be taken once a day. In addition, they will also receive measuring cups that denote a level of 4 ounces. The amount of BB-12 supplemented yogurt drink to be supplied will be 4 ounces per day, which is slightly more than 100 grams/day.

6.5.3 Randomization

The group allocation ratio is 2:1, 40 BB-12 to 20 control volunteers. The randomization scheme will be generated by the study statistician, Dr. Shuo Chen, using permuted blocks in random order: 4 blocks with the block size of 9 and 4 blocks with the block size of 6. It will be impossible for research personnel involved with participants to adjust randomization or discern what drinks participants were receiving, ensuring true allocation concealment. Participants who receive the control product will receive all identical interventions (i.e. stool collection, follow-up, etc.) that the BB-12 group receives.

Randomization will be completed after the participant consents to the study and completes the baseline assessment and run-in. The yogurt or antibiotic interventions may only be initiated after randomization.

6.6 Blinding

Each participant will be randomly allocated in a 2:1 ratio to either BB-12-supplemented yogurt or an identical yogurt without the added BB-12 probiotic. Both contain the starter culture YF-L702 (a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*). The yogurts are similar in taste and will have identical nutritional value, such as the same amount of calcium, protein, calories, etc. The yogurts are packed in identical packaging with six different bin numbers. The participants, PIs, research staff are all blinded to the treatment allocation.

The study statistician, Dr. Shuo Chen, will generate three group-labels (e.g. A, B, C) where two groups are the same, e.g. A=C: BB-12 group, B: control group. Dr. Chen will be blinded to which two are the two treatment groups and which one is the control group. These groups will be considered as three arms before unblinding.

PSU will send yogurt in six bins with distinct numbers, two of which will be control yogurt and four will be the BB-12 yogurt. Only the researchers at Maryland will know which bin numbers correspond to ‘group 1’ and which correspond to ‘group 2’, but will not know which is the active or control. PSU and Maryland have no contact with participants and will not release this information to the study team until the analyses are completed and databases are locked.

All study personnel at Georgetown and Dr. Chen will remain blinded throughout the study until the databases are locked. In the unfortunate event that a participant experiences a serious adverse event or emergency that requires unmasking the treatment groups, Dr. Ranit Mishori, a member of the Department of Family Medicine who is not affiliated with the study, will discuss the assignment and follow-up with the participant and their treating physician. Dr. Mishori has done this for our previous clinical trials.

6.7 Follow-up Visits

6.7.1 Telephone Follow-up

Follow-up phone visits will be completed on Days 7, 14 and 30 (±2 days). It is imperative that all visits be completed on a timely and regular fashion. Generally, the Follow-Up Form will be the only form completed during the phone calls, but at times depending on participant’s report, there may be another form or two. The follow up period includes all the time previous to the day of the interview. Any incidents that happen the day of the visit would be captured in the next Follow Up Visit.

There will be a set day that each subsequent visit should be completed for each participant. The research personnel should call all participant’s phone numbers at least one time per day in order to collect the data. If more than 3 business days have passed from the set day, the RA should alert the PI that this week’s Follow-Up Form has not yet been completed. If the RA is not able to contact the participant, a Missed Visit Report Form should be completed instead and the PI should be alerted once again.

If a participant is unmasked at any point in time during the study, the PI will be alerted immediately. A Georgetown University employee in the Department of Family Medicine not associated with the study will follow up with this participant from this point on. This particular research personnel is not to discuss any information regarding this occurrence with any other personnel other than the PI.

6.7.2 Telephone Follow Up Script

In general, the Day 7, 14 and 30 (±2 days) follow up will be administered over the phone, but may be completed in person. These phone visits are when the primary data will be collected. It is imperative that all visits be completed on a timely and regular fashion.

1) *Drink Bin Number:* Make sure the participant has the correct drink bin number as you are completing the Follow-Up Form.
   a) Ask the participant the number on the drink bottles and this should be confirmed with the randomization form.
   b) If this number is not consistent, the participant should be alerted not consume any more drinks until further notice and the project coordinator should be immediately alerted after completing this week's Follow-Up Form.

2) *Daily Assessment Diary:* Ask the participant to collect the Daily Assessment Diary for the phone conversation. A lot of the information that will be collected on the Follow-Up Form has been collected by the participant on their Daily Assessment Diary. If they have not been using the Daily Assessment Diary, please positively stress the importance of the Daily Assessment Diary.

3) Explain to participant that we are supposed to fill out this Follow-Up Form today and it should take about 5 minutes.

4) Administer Follow-Up Form.

- When entering sick days, missing school or work, etc., this should be generally whole integers. However, it is possible that a participant missed only 2 hours to go to the doctor. All numbers under 4 hours should be counted at 0.5 days, and all those above rounded up to next integer.
- The participant needs to decide if any activities were missed due to an illness. This is for all illnesses, even those that we are not collecting data about.
- For some questions the RA may say, "Did you visit a doctor?" A doctor is defined as any medical personnel in this study. Therefore, the participant may say, "Never, but s/he saw a nurse practitioner." Please count this the same as seeing the doctor.
- *Compliance:* Please let the participant know that we would like them to consume the interventions, diaries, and sample collections as per the schedule, but regardless, we appreciate their participation.
- *Adverse Events:* If the participant believes any AE occurred, it is imperative that the RA immediately fill out an AE Form, and if there are any SAE, alert the PI and project coordinator immediately.
- Confirm the date and time for the next visit or call.
- At day 7, remind the participant to take a photo of the remaining antibiotics bottles.
- At day 14, remind the participant to take a photo of the remaining yogurt bottle.

6.7.3 Completion/Final Evaluation

On the final follow-up at day 30, participants will provide the final sample and be queried for changes in baseline health and other adverse events.

7. SAFETY ASSESSMENTS

7.1 Specification of Safety Parameters

We will alert the participants of the common reactions to yogurt and by listing common reactions for them to monitor. We will also have close follow-up and 24-hour phone line accessibility there is a question of an allergic or idiosyncratic reaction.

Details of all adverse events reported by participants or observed by research personnel will be recorded in the adverse event report forms. All adverse events observed will be recorded separately and all will be reported to the IRB on IRB Adverse Event forms.

In order to prevent the occurrence of adverse events, we will be stringent in enforcing our exclusion criteria, paying particular attention to the potential for an allergic reaction to strawberry or yogurt on the part of the participant.

Any participants who develops evidence of allergy or hypersensitivity to any component of the investigational product will be withdrawn from the clinical study and receive no further doses of either drink. We will continue to follow these participants for safety for the remainder of the study.

7.2 Methods and Timing for Assessing, Recording, and Analyzing Safety Parameters

Unanticipated problems will be recorded in the data collection system throughout the study.

Details of all expected and unexpected adverse events reported by participants during follow-up calls, spontaneously, or observed by research personnel must be recorded in the adverse event report forms. Participants will be provided with a daily diary with a list of the most likely possible adverse events (see **Table 10**), where they can document expected and other unexpected events not already listed. All non-serious adverse events will be reported to the IRB during continuing review. All AEs regardless of severity level will be evaluated by the DSMB during scheduled meetings.

If a participant reports an AE/SAE that does not yet have a Date of Resolution (ongoing) and is continuous (no more than two days without interruption) across two follow-up periods, this AE/SAE will be considered one event. The total duration of the AE/SAE will be collected but only one AE/SAE report form will be completed for this particular event. If the symptoms resolved for more than two days before recurrence, these will be reported as separate events.

The study staff will record all reportable events with start dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. At each study visit, the study staff will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until satisfactory resolution or stabilization. In the event of SAEs, all clinical and laboratory investigations considered necessary by the investigator will continue until values have returned to normal. The investigator will provide the IRB with copies of results of examinations and treatment carried out during the follow-up of SAEs. These reports will be provided to the FDA and the NCCIH Program Official.

7.3 Adverse Events and Serious Adverse Events

An **adverse event (AE)** is any untoward medical occurrence in a participant during participation in the clinical study or with use of the experimental agent being studied. An adverse finding can include a sign, symptom, abnormal assessment (laboratory test value, vital signs, electrocardiogram finding, etc.), or any combination of these regardless of relationship to participation in the study. This includes events not

present at the start of the study, or worsening in grade during the course of the study. An AE can be reported anytime after study enrollment; a participant is considered enrolled once the informed consent form is signed.

A **serious adverse event (SAE)** is one that meets one or more of the following criteria:

- Results in death
- Is life-threatening (places the participant at immediate risk of death from the event as it occurred)
- Results in inpatient hospitalization or prolongation of existing hospitalization
- Results in a persistent or significant disability or incapacity
- Results in a congenital anomaly or birth defect

An important medical event that may not result in death, be life threatening, or require hospitalization may be considered an SAE when, based upon appropriate medical judgment, the event may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

Unanticipated Problems (UP): The Office for Human Research Protections (OHRP) considers unanticipated problems involving risks to participants or others to include, in general, any incident, experience, or outcome that meets **all** of the following criteria:

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied;
- Related or possibly related to participation in the research (“possibly related” means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

7.4 Characteristics of an Adverse Event

7.4.1 Relationship to Study Intervention

To assess relationship of an event to study intervention, the following guidelines are used:

1. Related (Possibly Related, Probably Related, Definitely Related)
   a. The event is known to occur with the study intervention.
   b. There is a temporal relationship between the intervention and event onset.
   c. The event abates when the intervention is discontinued.
   d. The event reappears upon a rechallenge with the intervention.

2. Not Related (Unlikely Related, Not Related)
   a. There is no temporal relationship between the intervention and event onset.
   b. An alternate etiology has been established.

7.4.2 Expectedness of SAEs

The Study PIs and Data Safety Monitoring Board (DSMB) will be responsible for determining whether an SAE is expected or unexpected. An adverse event will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the intervention.

Table 10. Possible expected adverse events and grading scale.[a]

CTCAE Term	Mild Grade 1	Moderate Grade 2	Severe Grade 3	Potentially Life Threatening Grade 4	Death Grade 5
Abdominal Pain (stomach ache)	Mild pain	Moderate pain; limiting instrumental ADL*	Severe pain; limiting self care ADL**	-	-
Allergic reaction	Systemic intervention not indicated	Oral intervention indicated	Bronchospasm; hospitalization indicated for clinical sequelae; intravenous intervention indicated	Life-threatening consequences; urgent intervention indicated	Death
Constipation	Occasional or intermittent symptoms; occasional use of stool softeners, laxatives, dietary modification, or enema	Persistent symptoms with regular use of laxatives or enemas; limiting instrumental ADL	Obstipation with manual evacuation indicated; limiting self care ADL	Life-threatening consequences; urgent intervention indicated	Death
Diarrhea (≥3 loose stools per day for 2 consecutive days)	Increase of <4 stools per day over baseline; mild increase in ostomy output compared to baseline	Increase of 4-6 stools per day over baseline; moderate ostomy output compared to baseline; limiting instrumental ADL	Increase of >=7 stools per day over baseline; hospitalization indicated; severe increase in ostomy output compared to baseline; limiting self care ADL	Life-threatening consequences; urgent intervention indicated	Death
Dyspepsia (upset stomach or indigestion)	Mild symptoms; intervention not indicated	Moderate symptoms; medical intervention indicated	Severe symptoms; operative intervention indicated	-	-
Fever	38.0 - 39.0°C (100.4 - 102.2°F)	> 39 - 40.0°C (102.3 - 104.0°F)	> 40.0°C (>104.0°F) for ≤ 24 hours	> 40.0°C (>104.0°F) for > 24 hours	Death
Flatulence	Mild symptoms; intervention not indicated	Moderate; persistent; psychosocial sequelae	-	-	-
Lack/loss of appetite	Loss of appetite without alteration in eating habits	Oral intake altered without significant weight loss or malnutrition; oral nutritional supplements indicated	Associated with significant weight loss or malnutrition (e.g., inadequate oral caloric and/or fluid intake); tube feeding or TPN indicated	Life-threatening consequences; urgent intervention indicated	Death
Loose stools (not clinically defined diarrhea)	Mild symptoms; intervention not indicated	Moderate symptoms; limiting instrumental ADL	Severe symptoms; limiting self care ADL; hospitalization indicated	Life-threatening consequences; urgent intervention indicated	-
Nausea	Loss of appetite without alteration in eating habits	Oral intake decreased without significant weight loss, dehydration or malnutrition	Inadequate oral caloric or fluid intake; tube feeding, TPN, or hospitalization indicated	-	-
Rash (Cutaneous reaction)	Covering <10% body surface area (BSA) with or without symptoms (e.g., pruritus, burning, tightness)	Covering 10-30% BSA with or without symptoms; limiting instrumental ADL; rash covering >30% BSA with or without mild symptoms	Rash covering >30% BSA with moderate or severe symptoms; limiting self care ADL	-	-
Vomiting	Intervention not indicated	Outpatient IV hydration; medical intervention indicated	Tube feeding, TPN, or hospitalization indicated	Life-threatening consequences	Death

[a] U.S. Department of Health and Human Services. Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0. Published: November 27, 2017.
Semi-colon (;) indicates 'or' within the description of the grade
Single dash (-) indicates grade is not available
Activities of Daily Living (ADL)
*Instrumental ADL refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.
**Self care ADL refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

7.4.3 Severity of Event

Adverse events will be graded according to the National Cancer Institutes' Common Terminology Criteria for Adverse Events (CTCAE) v5.0:

1. Grade 1 Mild: asymptomatic or mild symptoms; clinical or diagnostic observations only; no intervention indicated; no to minimal impact on activities of daily living (ADL).
2. Grade 2 Moderate: minimal, local, or noninvasive intervention indicated; moderate impact on ADL.
3. Grade 3 Severe: severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; needs major assistance with ADL.
4. Grade 4 Potentially life-threatening: life-threatening consequences; urgent intervention indicated.
5. Grade 5 Death: death related to AE.

Please note that a severe AE and an SAE are distinct terms.

7.5 Expectegd Risks

The expected risks and side effects (listed in **Table 10**) for each intervention and procedure are as follows:

- Cultured yogurt: digestive/gastrointestinal problems such as stomach ache, constipation, diarrhea, flatulence, fever, lack of appetite, vomiting, and loose, watery bowels, and allergic reaction to any of the yogurt ingredients, most likely strawberry.

- Amoxicillin-clavulanate: nausea and/or vomiting, diarrhea, upset stomach and mild to severe skin rash. The most serious potential risk is diarrhea caused by a *Clostridium difficile* infection. Symptoms of *C. difficile* infection include stomach pain or tenderness, bloody stools, fever, nausea, and diarrhea.

- Fecal sample collection: there are no known risks. However, there may be some discomfort with fecal sample collection.

These risks are considered to be minimal and are addressed in the protocol and consent form.
It will be explained to the participants that there may also be side effects, other than those listed, that we cannot predict. Many side effects go away shortly after the treatments are stopped, but in some cases side effects can be serious, long lasting or permanent. Participants will be informed to discuss any questions about potential risks with the researchers and their primary care providers. Anyone who develops evidence of allergy or hypersensitivity to any component of the yogurt products will be withdrawn from the study and receive no further doses. We will continue to monitor these participants for safety until the event sufficiently resolves.

7.6 Reporting Procedures

7.6.1 Unanticipated Problem Reporting

Incidents or events that meet the OHRP criteria for unanticipated problems require the creation and completion of an unanticipated problem report form. OHRP recommends that investigators include the following information when reporting an adverse event, or any other incident, experience, or outcome as an unanticipated problem to the IRB:

- Appropriate identifying information for the research protocol, such as the title, investigator's name, and the IRB project number;
- A detailed description of the adverse event, incident, experience, or outcome;

- An explanation of the basis for determining that the adverse event, incident, experience, or outcome represents an unanticipated problem;
- A description of any changes to the protocol or other corrective actions that have been taken or are proposed in response to the unanticipated problem.

To satisfy the requirement for prompt reporting, unanticipated problems will be reported using the following timeline:

- Unanticipated problems that are serious adverse events will be reported to the IRB, DSMB, and NCCIH within 7 days of the investigator becoming aware of the event.
- Any other unanticipated problem will be reported to the IRB, DSMB, and NCCIH within 14 days of the investigator becoming aware of the problem.
- All unanticipated problems should be reported to appropriate institutional officials (as required by an institution's written reporting procedures), the supporting agency head (or designee), and OHRP within one month of the IRB's receipt of the report of the problem from the investigator.

7.6.1 Adverse Event Reporting

SAEs that are unanticipated, serious, and possibly related to the study intervention will be reported to the DSMB, IRB, and NCCIH in accordance with requirements.

If any SAEs are reported, the PIs will be alerted immediately, as well as the IRB. If deemed necessary, members of DSMB will be alerted immediately. The PIs and/or IRB will decide if stopping criteria have been met. If they believe so, an ad hoc teleconference will be arranged by the DSMB Administrator. No other participants will be enrolled until after the teleconference, which will occur within 24 hours. Additionally, SAEs will be reported using the following timeline:

- Unexpected fatal or life-threatening AEs related to the intervention will be reported to the NCCIH Program Officer, and DSMB within 3 days of the investigator becoming aware of the event. Other serious and unexpected AEs related to the intervention will be reported within 7 days.
- Anticipated or unrelated SAEs will be handled in a less urgent manner but will be reported to the DSMB, IRB, and other oversight organizations in accordance with their requirements and will be reported to NCCIH on an annual basis.
- All other AEs documented during the course of the trial will be reported to NCCIH on an annual basis by way of inclusion in the annual report and in the annual AE summary which will be provided to NCCIH and to the DSMB. The DSMB Report will state that all AEs have been reviewed.

7.7 Follow-up for Adverse Events

The following applies only for non-serious adverse events; SAEs will follow the aforementioned policies for reporting and monitoring of serious adverse events.

For non-serious AE, if the AE has not resolved by the Day 14 follow-up, then depending on the type of AE (expected or unexpected) the research assistant will either continue to follow-up with the participant or consider Day 14 as the final Date of Resolution:

- If the AE reported is listed on the Adverse Event Report Form (Codes 1-12; as listed in **Table 10**) as a possible, expected AE, the research assistant will continue to follow-up the participant a minimum of every 4 days until the AE resolves to record the Date of Resolution.

- If the AE reported is listed as “Other” (Code 13), or unexpected, and the Principal Investigator AND participant considers the AE is not related/or is unlikely related to the intervention, the research assistant will not continue to follow-up past the Day 14 follow-up and will consider Day 14 as the final Date of Resolution.

7.8 Safety Monitoring

7.8.1 Data Safety Monitoring Board

The Data Safety Monitoring Board (DSMB) will function as an independent oversight committee charged with overseeing participant safety and providing recommendations to the PIs and Co-investigators regarding the continuing conduct of the study. The DSMB will review periodic blinded Safety Reports. Unblinded but sealed Safety Reports will be available to each DSMB member, should the committee determine that review of unblinded safety data is warranted.

The DSMB will include independent individuals not involved in this research study and is comprised of members Nicole Prewitt (community representative), Alexander Krist (physician), Geoffrey Preidis (probiotics and microbiome physician-scientist) and Chair, Elizabeth Carter (statistician). No member of the Committee has collaborated or co-published with the PIs within the past three years. They are qualified to review the patient safety data generated by this study because of their unique expertise. The clinical PI (Merenstein) and research team will provide the DSMB with an uptodate data set to ensure safety, effective data collection and progress. During the course of the study, the DSMB will meet regularly to review adverse events data and approve continuation of the protocol.

7.8.2 Safety Reports

Study progress and safety will be reviewed 25%, 50% and 75% data completion (and more frequently if needed). Progress reports, including patient recruitment, retention/attrition, and AEs will be provided to the DSMB. A Safety Report will be compiled and will include a list and summary of AEs. In addition, the reports will address (1) whether AE rates are consistent with pre-study assumptions; (2) reason for dropouts from the study; (3) whether all participants met entry criteria; (4) whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims of the study; and (5) conditions whereby the study might be terminated prematurely. The report will be sent to the DSMB and will be forwarded to the IRB and NCCIH. The IRB and other applicable recipients will review progress of this study on an annual basis.

The study team will generate Study Reports for the DSMB and will provide information on the following study parameters: the status of followups, adverse events and enrollment updates. Study Report tables will be generated only from aggregate (not by group assignment) baseline and aggregate safety data for the study population.

8. INTERVENTION DISCONTINUATION

Study enrollment and/or interventions may be temporarily suspended or terminated if:

- Any study participant experiences death that the DSMB, PIs or IRB believes may be related to the study interventions.
- If a statistically or clinically significant number of SAEs or hospitalizations occur that the DSMB believes may be related to the treatment.
- New information from outside studies reveals that it would be unethical to continue enrolling participants in the study.
- The IRB decides that the study should be stopped.
- If statistical differences among the primary outcomes will be determined that require stopping. For example, if the differences among certain interventions make it unethical to continue or if the effect size is so small that it is implausible a positive outcome could occur.

A safety review by the DSMB, IRB, and/or NCCIH will be convened to determine if the study should continue per protocol, proceed with enhanced monitoring, be further investigated, be discontinued, or be modified and then proceed. The FDA and NCCIH retain the authority to suspend additional enrollment and study interventions and administration of study product for the entire study, as applicable.

This study will be stopped prior to its completion if:
(1) the intervention is associated with adverse effects that call into question the safety of the intervention;
(2) difficulty in study recruitment or retention will significantly impact the ability to evaluate the study endpoints;
(3) any new information becomes available during the trial that necessitates stopping the trial; or
(4) other situations occur that might warrant stopping the trial.

Individuals may be withdrawn from the study for any of the following:
- Adverse events; the clinical PI (DM) will discuss AEs with the participant, and possibly with their personal physician, as needed, to determine whether the participant should be withdrawn from the study.
- If participant are deemed to be noncooperative (e.g. refuse to participate in follow-up calls or fail to complete diaries).
- If participants are lost to followup, i.e. more than 7 days without contact with study personnel during the follow-up period.
- Participant will not be withdrawn from the study solely for missed interventions or specimen collections, but may be withdrawn for non-cooperation or lost to follow-up.
- Serious adverse events related to the interventions, as determined by the PIs, participant or participant's physician.

9. STATISTICAL CONSIDERATIONS

9.1 General Design Issues

Primary Hypothesis: Antibiotics will result in a reduction in fecal SCFA. BB-12 supplementation will protect against antibiotic-induced SCFA (with acetate levels being used to represent the production of SCFA by gut microbiota) reduction and/or be associated with a more rapid return to baseline SCFA levels (acetate), compared to participants who receive a control yogurt without BB-12.

As reported by Hoverstad et al. (1986) and Clausen (1991), we hypothesize that antibiotic administration for 7 days will decrease mean fecal acetate levels for each participant, and that upon conclusion of antibiotic therapy fecal acetate levels will return toward baseline values for each participant.

We will compare the mean fecal acetate levels at days 7 and 30 versus the baseline values for participants receiving BB-12 or control yogurt. A significantly higher mean level of fecal acetate, calculated using one-way analysis of variance (Ganes-Howell *post hoc* test) in participants receiving BB-12 yogurt compared to the control yogurt during or after antibiotic administration, will be consistent with our hypothesis on the effect of BB-12, and will substantiate advancing the proposed study onto the next phase. As with the sample size calculation, we estimate a 42% decrease in acetate in the control/antibiotic treated group and no greater than a 15% decrease in the BB-12 treated group.

The following *biological signatures* will be assessed to determine if giving yogurt with BB-12 to humans results in a clinically meaningful change in these measures. We will move to the R33 phase if the R61 demonstrates the expected changes described for each signature, in the direction indicated:

a. BB-12 ameliorated antibiotic-induced decreases in acetate. We expect a minimal effect size for comparison of the acetate levels in the placebo group to the BB-12-treated group to have a Cohen's d ≥0.6. Effect size according to Cohen's d will be calculated at day 7 and day 30 with either day 7 or day 30 reaching Cohen's d ≥ 0.6 constituting an achieved milestone in the R61 Phase that will justify movement to the R33 Phase. Based upon the literature, we expect a 42% decrease in acetate with antibiotics and no greater than 15% decrease in acetate with BB-12, which would yield a Cohen's d >0.6.

Secondary Hypothesis: Antibiotics will result in a decrease in the overall number and diversity of bacterial species present in the fecal microbiota. BB-12 supplementation will protect against antibiotic-induced shifts in the microbiota and/or will be associated with a quicker return to a baseline microbiota composition, as compared to the control group. Microbiota composition will be assessed with 16S rDNA profiling and qPCR.

9.2 Sample Size and Randomization

Sample size calculations were informed by data from Hoverstad et al.,(120, 121) which compared changes in the mean SCFA levels between baseline fecal samples and fecal samples collected after six days administration of various antibiotics or placebo in healthy volunteers. In these studies, a mean reduction in acetate levels occurred from 45 ± 16 umol/g at baseline to 24 ± 18 umol/g after antibiotic treatment (46% reduction in acetate). Reductions in acetate after antibiotic treatment ranged from 5% to 79% with ampicillin, a similar antibiotic to amoxicillin.(120, 121) We can infer similar decreases in acetate in our proposed study. Using a conservative estimate of a 42% decrease in acetate in the control/antibiotic treated group and no greater than a 15% decrease in the BB-12 treated group, and an allocation ratio of 2:1 (BB-12 to control volunteers); a total sample size of 60 participants is necessary to have 80% power to detect a significant difference in mean acetate levels as a measure of gut microbiota production of SCFA between a control and BB-12-treated group.

With a sample size of 40 subjects in the active arm and 20 subjects in the control arm and attrition rate of 5% at day six and 20% at day 30, we would have power of 82% to detect a moderate effect size of Cohen's d = 0.63 (assuming the intra-correlation correlation coefficient is 0.3). The α level of 0.05 will be used for the analysis. Since the effect size of previous study (reduction in acetate levels) is Cohen's d =1.23, we should have ample power to detect the treatment effect. (The power calculation is based on the R software and packages simr, sjstats, and pwr).

9.2.1 Treatment Assignment Procedures

The group allocation ratio is 2:1, 40 BB-12 to 20 control participants. See Section 6.5.3 on Randomization.

The web-based system will integrate the randomization scheme and automatically assign enrolled participants to the corresponding allocation. All research personnel will be naive to group assignments

9.3 Definition of Populations

Statistical analysis on the primary outcome will be performed using the intention to treat principle; all subjects enrolled and randomized will be accounted for in the final analysis in their randomized group.

We will also examine the per protocol group separately and report both the intention-to-treat and per protocol analyses. The mixed effect model will be used, which is compatible with unbalanced outcome measurements. The multiple imputation analysis will be performed when the covariates are missing.

9.4 Interim Analyses and Stopping Rules

Since this is a mechanistic study, analyses of primary and secondary outcome data will not be conducted before the study is completed. The main purpose is to examine the effects of the treatment of BB-12 (on SCFA and microbiome changes), and thus non-inferiority or bio-equivalence testing is not indicated. There will be no interim or futility analysis due to mechanistic aims.

9.5 Outcomes

9.5.1 Primary Outcome

The primary outcome is a difference, measured at day 7, in antibiotic-induced changes in fecal SCFA (with a particular focus on acetate) in participants receiving BB-12-supplemented yogurt versus non-supplemented control yogurt.

9.5.2 Secondary Outcomes

The secondary outcome is a difference, measured at days 7 and 14, in antibiotic-induced changes in the number and diversity of bacteria in the gut microbiota.

9.6 Anticipated Results

The most likely outcomes from the study are:

1. +/- impact of BB-12 on fecal SCFA levels
2. +/- impact of BB-12 on gut microbiota composition (measured to quantify level of disruption achieved by antibiotics as a comparator within this study)
3. +/- impact of BB-12 on AAD (measured, but study not powered to detect clinical difference of AAD).

If BB-12 is found to have a positive effect on the biological signature (acetate levels) as compared to the control, the work to be carried out in the R33 will replicate the R61 methods in a second cohort of

patients, with the addition of timing of probiotic administration, as well as expanding the analysis to characterize the functional microbiome using metatranscriptomic methods. This information will provide a framework for future research in the characterization of other probiotic strains for AAD.

Negative overall group results, i.e. no significant difference between the control and BB-12 groups with respect to longitudinal changes in acetate levels and microbiome composition versus baseline values, will suggest that BB-12 does not impact the gut environment via the mechanisms we hypothesized. However, based on past studies of AAD and BB-12, it is unlikely that we will see no responders to this natural product. However, based on past studies of AAD and BB-12, it is unlikely that we will see no effect of the BB-12 probiotic on maintenance of acetate levels when administered concurrently with antibiotic. Thus, we intend to correlate the changes in acetate levels, by examining the highest and lowest quartiles over time in study participants receiving probiotic to assess whether we can predict the impact of BB-12 using the baseline. Such results will enable fine-tuning of conclusions based on individual responses and suggest new hypotheses for future studies.

9.7 Data Analyses

To examine the primary hypothesis that BB-12 supplementation will protect against antibiotic-induced SCFA reduction, the mixed model will be used to analyze the treatment effect on the antibiotic-induced SCFA reduction. Statistical analyses will be performed using intent-to-treat analytic strategies. The mixed effect model will be used, which is compatible with unbalanced outcome measurements. The change of SCFA from baseline will be considered as the outcome variable of the mixed model. All time points will be included for the analysis. Covariates including age, sex, BMI and time (a continuous variable) will be adjusted when necessary. In addition, the time and treatment interaction term will be assessed by the likelihood ratio test. The random intercept will be used and the AR1 and/or compound symmetry covariance structure will be considered (and then the likelihood test can help to determine the more appropriate covariance structure). The final model selection will be performed based on information criteria such as Akaike information criterion (AIC) or Bayesian information criterion (BIC). Power: with a sample size of 40 subjects in the treatment arm and 20 subjects in the placebo arm and attrition rate of 5% at day six and 20% at day 30, we would have power of 82% to detect a moderate effect size of Cohen's d = 0.63 (assuming the intra-correlation correlation coefficient is 0.3). The α level of 0.05 will be used for the analysis. Since the effect size of previous study (reduction in acetate levels) is Cohen's d =1.23, we should have ample power to detect the treatment effect. (The power calculation is based on the R software and packages simr, sjstats, and pwr).

9.8 Missing Data

We understand the importance of limiting missing data and as in previous studies the PI and project coordinator will work very closely to prevent missing data. However, there will be some missing data, which we anticipate will be missing at random (MAR). Although it is challenging to test the assumption MAR versus missing not at random (random) due to incomplete information for such test (122), we will carefully examine the missing patterns and perform sensitivity analysis for the MAR assumption (123). The multiple imputation will be used to deal with the missing data (e.g. the missing covariate and baseline outcome variable).

A sensitivity analysis could be used to explore the impact of departures from the MAR assumption underlying the main analysis. For example, we let the mean of observed data and the mean of the unobserved data differ from each other, adjusted for other observed variables. Under an MAR analysis, *the difference* is assumed to be zero. We then evaluate the impact of difference on treatment effect inference under MAR assumption.

To test the secondary hypothesis, we will examine the treatment effects on multiple microbiota composition scores, for example, Bray-Curtis similarity score, Chao1 metric (α-diversity or richness), the Shannon index. We will apply mixed model to analyze these metrics and then perform multiple testing adjustment using FDR and/or FWER correction. The secondary hypothesis analysis is primarily exploratory and will not be used for Go/No Go for R33.

10. DATA COLLECTION AND QUALITY ASSURANCE

10.1 Data Collection Forms

Blinded research assistants will be collecting participant information in person or over the phone, and will be responsible for picking up samples from participants. All investigators and research personnel will take required classes on human subject research and the protection of individually identifiable health information prior to any contact with the participants.

Participants will be completing questionnaires with protected health information, and the release of confidential information is a risk. The research staff will take all necessary precautions to minimize this risk. Participants' addresses and phone numbers will be collected, as will other private health information. In accordance with the appropriate HIPAA regulations, all information will be protected and kept confidential at all times. All contact and study information will be kept on password-protected computers or online databases, participants will be identified by code, and no information will be released without proper approvals or permissions.

10.2 Data Management

Data management is a joint effort between the study team, PIs, and biostatistician. The investigators are responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported. All source documents and data should be should be attributable, legible, contemporaneous, original, accurate and complete (ALCOAC). The investigators will maintain adequate case histories of study participants, including accurate case report forms (CRFs), and source documentation.

10.2.1 Data Collection

Data collection and accurate documentation are the responsibility of the study staff under the supervision of the investigator. All source documents and laboratory reports are maintained by the study team, who will ensure that they are accurate and complete. Unanticipated problems and adverse events must be reviewed by the investigator or designee.

10.2.2 Data Management System

This study uses a project database within the Remote Electronic Data Capture (REDCap) system hosted by MedStar Health Research Institute (MHRI), and is FDA 21 CFR Part 11 compliant. The database will be secured with individual account logins and password protection. Each user must use their unique, assigned user account and password.

The database incorporates an electronic audit trail to show change(s) to data after original entry including the date/time and user making the change. REDCap has some builtin validation processes to ensure data quality, including minimum/maximum ranges, data type, calculations, date, and branching logic fields.

The data entry system is designed to allow information contained on a single form to be entered over one or multiple keying sessions. For direct electronic capture, it is recommended to enter any one form in its entirety rather than breaking up a single form into multiple keying sessions. Once an entry is created, any later additions or changes to incomplete forms or sessions will require the user to log a 'change reason'. The data collection forms were also designed for keying directly into the data entry system without the need for additional transcription.

Entries in REDCap may be saved locally on the device and then backed up on the server once the device is online. In the case of an outage of the REDCap system, a backup paper form will be used to collect the data, and will be entered into the database at a later time.

The biostatistician will receive only coded information that is entered into the database under study identification numbers. Electronic communication with outside collaborators will involve only de-identified information.

10.2.3 Source Documentation

Source documents are original documents, data, and records (e.g., hospital records, clinical and office charts, laboratory reports, memoranda, diaries or evaluation checklists, or pharmacy dispensing records) which first records a finding, observation, or other activity during a study.

Since this study will enroll generally healthy participants and data are entered directly into the electronic database, the case report and data collection forms will serve as the source documents. Any hardcopy participant study records, such as case report forms, medical records (if applicable), and participant files, are kept in a locked cabinet inside a locked office. Electronic records, identified by study ID code only, are stored in the REDCap database or on Georgetown University secure servers, both of which require a username and password. Only authorized, trained research staff will have access to the data.

Source Document/Form	Abbreviation	Key Form	Completed by
Baseline Health Status	BH	X	Research staff
Daily Assessment Diary (Days 1-7, 8-14)	DD	X	Participant
Demographic Information	DI	X	Research staff
Automated Self-Administered 24-Hour (ASA24®) Dietary Assessment Tool	ASA24		Research staff
Follow-up Form	FU	X	Research staff
Gastrointestinal Symptom Rating Scale - IBS	GS		Research staff
Inclusion/Exclusion	IE		Research staff
Informed Consent Process Documentation	IP		Research staff
Post Run-In Health Status	PR	X	Research staff
Pre-Screening	PS		Research staff
Randomization	RZ		Research staff
Sample Collection	SC		Research staff
Event-driven forms			
Adverse Event Report	AE	X	Research staff
Diarrhea Report and Follow-up	DR		Research staff
Missed Contact Report	MC		Research staff
Serious Adverse Event Report	SA	X	Research staff
Withdrawal from Intervention	WI		Research staff

Other source documents may include study progress notes, electronic or paper medical records, laboratory reports, memoranda, diaries, and other records pertaining to the participant while in the research study.

10.2.4 Study Records Retention

Study documents will be retained for a minimum of 3 years following the formal discontinuation of clinical development of the investigational product, unless the funder specifically requests otherwise.

10.3 Quality Assurance

10.3.1 Training

All study staff will complete training on appropriate data collection and entry procedures, as well as the study specific databases. Completed trainings will be recorded in the training log, which is on file in the study regulatory binder. All key personnel (all individuals responsible for the design and conduct of this study) have completed Human Subjects Research Protection Training.

10.3.2 Quality Control

The PI of the recruitment site, Dr. Merenstein, is responsible for quality management for this study. The Project Coordinator has been designated by Dr. Merenstein to be responsible for the implementation of the quality management plan.

All source documents and case report forms are to be completed in a manner to ensure the data is attributable, legible, contemporaneous, original, accurate and complete. ALCOAC applies to both paper and electronic source data, and the records that hold that data. The REDCap system uses an electronic audit trial and signature capability to ensure each addition or change in the database is attributable to a unique user, when it was added/changed, the reason for the change, and when the review of the record is completed.

10.3.3 Metrics

Data validation and quality reports will be generated on a monthly basis. This report will include validation errors and other discrepancies in the data. The study staff will be responsible for resolving errors and discrepancies by checking the source documents and making the necessary changes in the database. Discrepancies should be resolved within 10 business days.

Performance reports will be sent to the Dr. Merenstein on a monthly basis. These will report on performance trends, i.e. enrollment status compared to enrollment goals, rates of missed visits, lost to follow-ups, and missing data. The PIs can use these reports to gauge how the study team is performing in the study.

10.3.4 Protocol Deviations

A protocol deviation is any noncompliance with the clinical trial protocol or Good Clinical Practice (GCP) requirements. The noncompliance may be either on the part of the participant, the investigators, or the study personnel. As a result of deviations, corrective actions are to be developed by the site and implemented promptly. It is the responsibility of the study personnel to use continuous vigilance to identify protocol deviations. All deviations from the protocol must be addressed in study subject source

documents. Protocol deviations must be sent to the local IRB per their guidelines. The PIs/study personnel will be responsible for knowing and adhering to their IRB requirements.

10.3.5 Monitoring

During the first year of recruitment, an internal source verification and file audit will be conducted on three randomly selected participant records. Study personnel will not be notified of the three charts in advance and must be prepared to pull all source documents. The audit is expected to last one day and will be conducted by research division staff member who is not part of the core study team.

During the audit, the study databases will be compared with the source documents, and the reviewer will check that all participants have signed informed consents, verify protocol procedures and records, and ensure that the research personnel have uptodate study documentation available. The reviewer will provide a written report of all findings to the PI within 10 business days of the audit. The PI will send a written response within 10 business days of receiving the audit report to document the response and corrective action plans. Significant protocol deviations or issues will be added to the next DSMB report.

11. PARTICIPANT RIGHTS AND CONFIDENTIALITY

11.1 Institutional Review Board (IRB) Review

Prior to enrollment of subjects into this trial, the approved protocol and the informed consent form as well as all internal and external advertisements will be reviewed and approved by the Georgetown University IRB. The Georgetown University IRB operates under the appropriate Federal Regulations, and the research team have taken required classes on human subject research, ethics and HIPAA.

The responsible official at the Georgetown University IRB will provide the IRB letter of approval of the protocol prior to the start of this trial. Any amendments to the protocol will be submitted to the IRB and approval obtained prior to implementation.

11.2 Informed Consent Forms

Informed consent is a process that is initiated prior to an individual's agreeing to participate in the study and continuing throughout an individual's study participation. Extensive discussion of risks and possible benefits of this therapy will be provided to the participants. Consent forms describing in detail the study interventions/products, study procedures, and risks are given to the participants and written documentation of informed consent is required prior to starting intervention/administering study product. The research team will discuss all information outlined in the informed consent document with prospective participants to ensure that they understand the nature of the research and can voluntarily decide whether or not to participate.

After screening, research personnel will meet with the participant and administer the informed consent process. Dr. Merenstein will review, sign and date each consent form after the participant signs it.

11.3 Participant Confidentiality

Participant confidentiality is strictly held in trust by the investigators, their staff, and the sponsor(s) and their agents. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. In accordance with the appropriate HIPAA regulations, all information will be protected and kept confidential at all times. All hardcopy participant study records, such as case report forms, will be kept in a locked cabinet inside a locked office. All contact and study information will be kept on password-protected computers or online databases, participants will be identified by code, and no information will be released without proper approvals or permissions.

The FDA, study monitor, or other authorized representatives of the sponsor may inspect all documents and records required to be maintained by the investigators. The clinical study site will permit access to such records.

11.4 Study Discontinuation

The study may be discontinued at any time by the IRB, the NCCIH, the OHRP, the FDA, or other government agencies as part of their duties to ensure that research participants are protected.

11.5 Ethical Standard

The investigator will ensure that this study is conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research of the US National Commission for the Protection of Human Subjects of Biomedical and Behavioral

Research (April 18, 1979) and codified in 45 CFR Part 46 and the ICH E6; 62 Federal Regulations 25691 (1997).

12. COMMITTEES

The Data Safety Monitoring Board (DSMB) will function as an independent oversight committee (see Section 7.8).

13. PUBLICATION OF RESEARCH FINDINGS

Investigators will make publication decisions jointly, consistent with their respective institutional and NIH policies. The respective research administration offices of the institutions will be responsible for preparing and negotiating an agreement for the conduct of the research and disposition of any resulting intellectual property, consistent with their respective institutional policies and NIH policy.

Efforts will be made to publish our research findings in peer-reviewed, scientific journals in a timely manner. All final peer-reviewed manuscripts that arise from this proposal will be submitted to PubMed Central in accordance with the NIH Public Access Policy. Additionally, we will follow any publication policies outlined in the Notice of Award provided by NCCIH.

This trial is registered at the NIH trials registry (http://ClinicalTrials.gov). Reporting of results of this trial will be in accordance with the recommendations of the CONSORT statement (http://www.consort-statement.org) and International Committee of Medical Journal Editors (http://www.icmje.org/).

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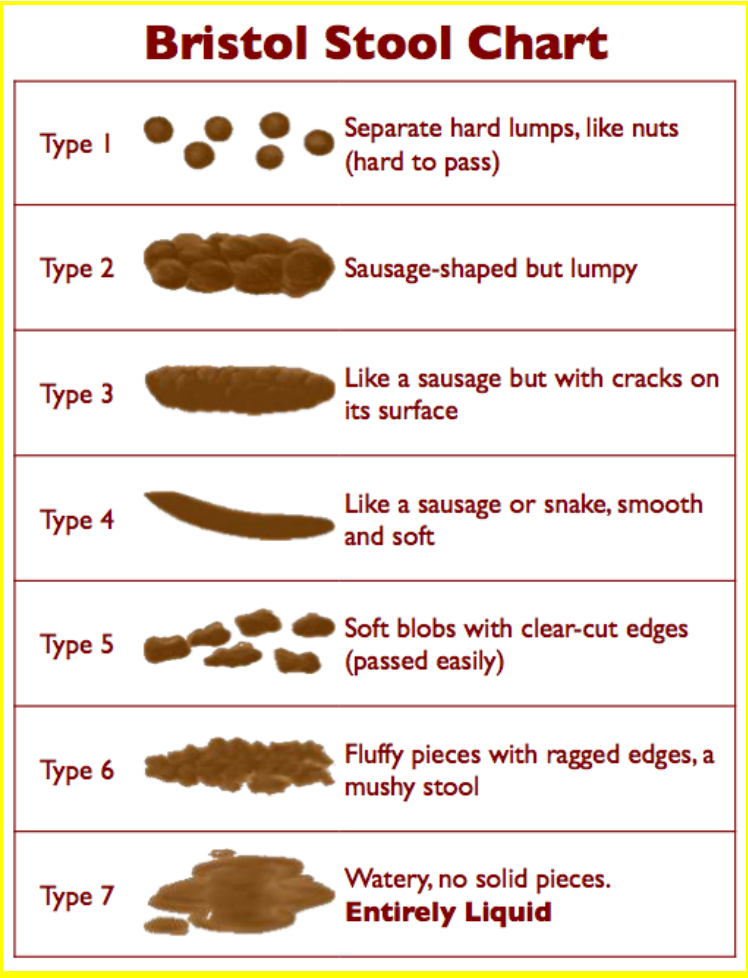
15. SUPPLEMENTS/APPENDICES

APPENDIX 1: Bristol Stool Chart



Heaton, K W & Lewis, S J 1997, 'Stool form scale as a useful guide to intestinal transit time'. *Scandinavian Journal of Gastroenterology*, vol.32, no.9, pp.920 - 924. Retrieved on 2/3/2007.

APPENDIX 2: Summary of BB-12 Safety Articles Using the Identical Strain

A) Langhendries, J.P., et al., *Effect of a fermented infant formula containing viable bifidobacteria on the fecal flora composition and pH of healthy full-term infants.* J Ped Gastro Nutr, 1995. **21**(2): p. 177-81.
In a double-blind, randomized controlled three-armed study, 54 healthy infants were breast-fed, given a fermented infant formula containing BB-12, or fed a non-acidified formula during the first two months of life. Growth, tolerance, acceptability and fecal flora were assessed. **No adverse events were reported.** The BB-12 formula was found to be well-tolerated and promoted normal growth during the first two months of life, as compared to the breast-fed infant group

B) Haschke, F., et al., *Clinical trials prove the safety and efficacy of the probiotic strain Bifidobacterium Bb12 in follow-up formula and growing-up milks*. Monatsschr Kinderheilkd (Suppl 1), 1998. 146: p. S26-S30.
Double blind, randomized, controlled feeding studies with BB-12 in the U.S., China, and Thailand demonstrated normal growth of healthy children between 4 and 36 months of age and catch-up growth in malnourished children. Feeding the milk-based formula with BB-12 resulted in protection from rotavirus infection, fewer periods with hard bowel movements and a lower incidence of diaper rash. **No adverse events were reported.**

C) Saavedra, J.M., et al., *Long-term consumption of infant formulas containing live probiotic bacteria: tolerance and safety.* Am J Clin Nutr, 2004. **79**(2): p. 261-7.
In a double-blind, randomized placebo controlled study, 118 healthy infants age 3-24 months consumed formula for 210±127 days. Thirty-nine infants received a standard milk-based formula supplemented with a high dose of BB-12 and TH-4, 39 infants received a low supplementation of BB-12 and TH-4 and 40 infants received an unsupplemented formula. The supplemented formulas were well accepted and were associated with a significant lower frequency of reported colic or irritability ($p<0.001$) and a lower frequency of antibiotic use ($p<0.001$) than unsupplemented formula. Of the 5 infants who developed an intercurrent illness or complaint, 4 had vomiting and diarrhea (1 in the placebo group and 3 in the high dose group) and 1 (high dose group) had otitis media. Three infants ended their participation because of an effect perceived by their parents to be related to formula consumption. Of these three, one infant developed a rash (after consuming the formula for 30 days), but was diagnosed as being viral by the pediatrician. The second infant had loose stools and the third had loose stools and vomiting (both in the high dose group). **Results showed no significant differences between groups in growth, health care attention seeking or other health variables, and therefore can be concluded that long-term consumption of BB-12 and TH-4 supplemented formulas are well tolerated and safe.**

D) Bin-Nun, A., et al., *Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates.* J Pediatr, 2005. **147**(2): p. 192-6.
In this study, 145 neonates of less than or equal to 1500g birth weight were given a daily feeding of a BB-02, BB-12 and TH-3 supplemented probiotic mixture or were given no feed supplements. Using Bell's criteria, development of necrotizing enterocolitis (NEC) was assessed. There were no differences in the incidence of any signs of feeding intolerance such as diarrhea, abdominal distension, or vomiting between the study and control groups. Three infants in study group versus eight infants in the control group died (P=.218; RR 0.38; yielding a relative risk reduction of 62%; 95% CI: 0.38-1.38). The three deaths in the control group were attributed to NEC, whereas there were no NEC-associated deaths among the treated infants. There were no other significant differences in the characteristics of those who died between the two groups. **No other adverse events were reported.**
E) Weizman, Z. and A. Alsheikh, *Safety and tolerance of a probiotic formula in early infancy comparing two probiotic agents: a pilot study*. J Am Coll Nutr, 2006. **25**(5): p. 415-9.

Fifty-nine full-term infants aged 3-65 days were randomly assigned for 4 weeks to a standard milk-based formula supplemented with either BB-12, Lactobacillus reuteri (ATCC 55730) or a probiotics-free formula. The supplemented formulas were well accepted and did not reveal any adverse effects. A comparison of growth parameters, and variables of feeding, stooling, crying and irritability did not reveal any significant differences between groups. No adverse events were reported in any of the participants. Therefore, use of formula supplemented with either BB-12 or Lactobacillus reuteri in early infancy, was safe, well tolerated and did not adversely affect growth, stooling habits or infant behavior.

F) Saavedra, J.M., et al., *Feeding of Bifidobacterium bifidum and Streptococcus thermophilus to infants in hospital for prevention of diarrhoea and shedding of rotavirus*. Lancet, 1994. **344**(8929): p. 1046-9.
In a double-blind, placebo-controlled trial, 55 hospitalized infants aged 5-24 months were fed either a formula supplemented with probiotics Bifidobacterium bifidum and Streptococcus thermophilus or a formula without probiotics. Subjects were evaluated for diarrhea and rotavirus shedding for 4447 patient-days during 17 months. All infants maintained or improved their nutritional status throughout the study. There were no adverse effects judged to be associated with the feeding of either formulas, and adequate growth was recorded in all subjects. The probiotic formula was well tolerated by the infants, many of whom were initially malnourished or immunocompromised.

G) Sheu, B.S., et al., *Impact of supplement with Lactobacillus- and Bifidobacterium-containing yogurt on triple therapy for Helicobacter pylori eradication.* Aliment Pharmacol Ther, 2002. **16**(9): p. 1669-75.
One hundred and sixty Helicobacter pylori-infected patients were randomized to receive either 1 week of triple therapy without supplements or triple therapy with Bifidobacterium BB-12 and Lactobacillus acidophilus LA-5 yogurt (AB-yogurt). In the triple-plus-yogurt group, AB-yogurt was continued for 4 weeks after completion of triple therapy. **Common side-effects, such as vomiting, constipation, diarrhea and metallic taste, were significantly decreased in the triple-plus-yogurt group**, with 15 events reported in the triple-plus-yogurt group and 53 reported in the triple-only group. In summary, supplementation with AB-Yogurt can improve drug compliance and thus enhance the intention-to-treat eradication rate of H. pylori after triple therapy. Furthermore, continued supplementation with AB-Yogurt after triple therapy can restore Bifidobacterium in stools after 4 weeks.

H) Phuapradit, P., et al., *Reduction of rotavirus infection in children receiving bifidobacteria-supplemented formula.* J Med Assoc Thai, 1999. **82 Suppl 1**: p. S43-8.
One hundred and seventy-five children aged 6-36 months were randomized to receive either a milk based follow-up formula, formula supplemented with BB-12 alone or formula supplemented with BB-12 and Streptococcus thermophilus for a period of 8 months. There were 81 episodes of diarrhea during the study period, most of which were caused by bacterial enteropathogens and only 3 were of rotavirus origin. The caregivers of the participants reported no adverse events.

I) Laake, K.O., et al., *Assessment of mucosal inflammation and circulation in response to probiotics in patients operated with ileal pouch anal anastomosis for ulcerative colitis.* Scand J Gastroenterol, 2003. **38**(4): p. 409-14.
Ten patients operated with ileal-pouch-anal anastomosis for ulcerative colitis were given a fermented milk product containing live lactobacilli (La-5) and bifidobacteria (BB-12) daily for 4 weeks. The results suggested that probiotics primarily act superficially, with change of gross appearance of the mucosa at endoscopy, but without significant effect on histological picture, mucosal perfusion or faecal calprotectin, during a relatively short period of 4 weeks. **No adverse effects were recorded.**
J) Anderson, A.D., et al., *Randomised clinical trial of synbiotic therapy in elective surgical patients.* Gut, 2004. **53**(2): p. 241-5.
In a double blind, randomised placebo controlled study, 137 patients for elective laparotomy were

enrolled 2 weeks prior to receive a combination of pre- and probiotics (synbiotic) Lactobacillus acidophilus La5, Bifidobacterium lactis BB-12, Streptococcus thermophilus, and Lactobacillus bulgaricus, together with the prebiotic oligofructose or a treatment of placebo capsules and sucrose powder. Seventy-two patients were in the synbiotic group and 65 patients in the placebo group. **There were no differences in the incidence of septic morbidity between the placebo and synbiotic groups.** The most common sites of infection were the urinary tract, respiratory tract and surgical wound.

K) Anderson, A.D., et al., *Randomized clinical trial of multimodal optimization and standard perioperative surgical care.* Br J Surg, 2003. **90**(12): p. 1497-504.
Twenty-five patients undergoing colonic resection were randomized into two groups receiving a ten-point optimization program or standard care. Optimized care included pre- and probiotic treatments for 7–14 days before surgery. The prebiotic used was oligofructose and the probiotic capsules contained Lactobacillus acidophilus La5, Lactobacillus bulgaricus, Bifidobacterium lactis BB-12 and Streptococcus thermophilus. Five patients in the conventional group reported 6 instances of postoperative complications: urinary retention (one patient), atrial fibrillation (two), respiratory depression related to PCA (two) and ileus (one); compared to four patients reporting of 5 instances of complication in the optimization group: ineffective epidural (two patients), ileus (one), urinary tract infection (one) and wound infection (one). **No adverse events inconsistent with post-operative recovery were reported.** One patient in the control group died from a perioperative myocardial infarction on the first postoperative day. In conclusion, optimization of surgical care significantly improved patients' physical and psychological function in the early postoperative period and facilitated early hospital discharge.

L) Mohan, R., et al., *Effects of Bifidobacterium lactis Bb12 supplementation on intestinal microbiota of preterm infants: a double-blind, placebo-controlled, randomized study.* J Clin Microbiol, 2006. **44**(11): p. 4025-31.
A double blind, placebo-controlled, randomized clinical trial was performed on 69 preterm infants born with a gestational age of <37 weeks. The infants were randomized into two groups to receive a probiotic formula supplemented with BB-12 or a placebo formula from the first day after birth for 21 days. **No adverse effects were observed in any of the infants.**

M) Pitkala, K.H., et al., *Fermented cereal with specific bifidobacteria normalizes bowel movements in elderly nursing home residents. A randomized, controlled trial*. J Nutr Health Aging, 2007. **11**(4): p. 305-11.
In a randomized, double blind, placebo-controlled trial, 209 elderly nursing home residents were randomized to received daily a fermented oat drink with either 1) Bifidobacterium longum strains or 2) BB-12 or 3) without viable bacteria (placebo) for 7 months. No adverse events were reported. The fermented oat drinks were well taken and well tolerated by subjects, with compliance at 85%.

N) Saarela, M., et al., *Tetracycline susceptibility of the ingested Lactobacillus acidophilus LaCH-5 and Bifidobacterium animalis subsp. lactis Bb-12 strains during antibiotic/probiotic intervention.* Int J Antimicrob Agents, 2007. **29**(3): p. 271-80.
In this study, 10 patients suffering from respiratory tract infections consumed doxycycline (tetracycline) for 10 days together with probiotic capsules containing Lactobacillus acidophilus La-5 and Bifidobacterium Bb-12. As a control group, 10 volunteers consumed only probiotic capsules for 14 days. **No adverse events were reported.**

O) Matsumoto, M., et al., *Effect of Bifidobacterium lactis LKM 512 yogurt on fecal microflora in middle to old aged persons*. Microbial Ecology in Health and Disease, 2000. **12**: p. 77-80.
In this study, eleven long-term inpatient volunteers aged 50-93 years, consumed a yogurt product

containing Bifidobacterium lactis LKM 512 (BB-12), Lactobacillus bulgaricus LKM 1759 and Streptococcus thermophilus LKM 1742 daily for 2 weeks. The study lasted 7 weeks with the following schedule: pre-administration (1 week), probiotic-supplemented yogurt administration (2 weeks), post-administration period (2 weeks) and administration of ordinary yogurt without probiotics (placebo yogurt) (2 weeks). **No adverse events were reported.**

P) Laake, K.O., et al., *Influence of Fermented Milk on Clinical State, Fecal Bacterial Counts and Biochemical Characteristics in Patients with Ileal-Pouch-Anal-Anastomosis.* Microbial Ecology in Health and Disease, 1999. **11**: p. 211-17.
In a double-blind, randomized, controlled study, 16 patients with restorative proctocolectomy for ulcerative colitis randomized to two groups: 8 patients ingested 500 ml of a fermented milk product containing live LA-5 and BB-12 (in its regular form) and 8 patients ingested 500 ml of a heat-treated form of the same product for one week. **No adverse effects were recorded.**

Q) Matsumoto, M., et al., *Impact of LKM512 yogurt on improvement of intestinal environment of the elderly*. FEMS Immunology and Medical Microbiology, 2001. **31**: p. 181-86.
In a placebo controlled study, 6 elderly volunteers of average age 78 years, consumed BB-12 yogurt then consumed a placebo yogurt without BB-12 supplementation. Fecal samples were tested before and after administration of both products as well as at Week 1 and Week 2 during administration. It was observed that the intestinal environment of the volunteers improved after the ingestion of BB-12 yogurt. **No adverse effects were reported.**

R) Jain, P.K., et al., *Influence of synbiotic containing Lactobacillus acidophilus La5, Bifidobacterium lactis Bb 12, Streptococcus thermophilus, Lactobacillus bulgaricus and oligofructose on gut barrier function and sepsis in critically ill patients: a randomised controlled trial.* Clin Nutr, 2004. **23**(4): p. 467-75.
The aim of this study was to determine whether the oral administration of a synbiotic preparation could alter gut barrier function in critically ill patients and thus reduce sepsis. Ninety patients admitted to an intensive care unit were randomised to receive either synbiotic or placebo preparations (45 into each group). The synbiotic preparation consisted of Lactobacillus acidophilus La5, Bifidobacterium lactis BB-12, Streptococcus thermophilus and Lactobacillus bulgaricus (probiotics), in dose of one capsule three times a day, with oligofructose (prebiotic), administered twice a day. The median duration of intake of study medication was 10 days in both groups. Fifty-nine patients developed one or more septic complication during the study; 33/45 (73%) in the synbiotic group and 26/45 (58%) in the control group. **This did not represent a statistically significant difference** (χ^2 2.41, P=0.12). The mortality rate was similar in both groups; 49% (22/45) in the synbiotic group and 45% (20/45) in the control group (χ^2 0.17, P=0.672).

S) Laake, K.O., et al., *Assessment of mucosal inflammation and blood flow in response to four weeks' intervention with probiotics in patients operated with a J-configurated ileal-pouch-anal-anastomosis (IPAA).* Scand J Gastroenterol, 2004. **39**(12): p. 1228-35.
Forty-one patients with ulcerative colitis and ten patients with familial adenomatous polyposis operated with ileal-pouch-anal-anastomosis were given 500 ml of a fermented milk product (Cultura) containing BB-12 and LA-5 daily for 4 weeks. The aim of the study was to determine, in conjunction with an earlier study, if mucosal perfusion in the distal part of the ileal pouch is reduced in patients on probiotic intervention. All patients completed the treatment period and reported intake of Cultura according to the study protocol **with no report of adverse effects.**

T) Laake, K.O., et al., *Outcome of four weeks' intervention with probiotics on symptoms and endoscopic appearance after surgical reconstruction with a J-configurated ileal-pouch-anal-anastomosis in ulcerative*

colitis. Scand J Gastroenterol, 2005. **40**(1): p. 43-51.
In a previous double-blind, randomized, controlled study, clinical improvement of symptoms was demonstrated in patients with ulcerative colitis (UC) operated on with ileal-pouch-anal-anastomosis (IPAA), during intervention with live probiotic microbes Lactobacilli and Bifidobacteriae. The aim of the present study was to confirm the previous results in a larger population, including clinical symptoms, fecal flora and endoscopic evaluation, and to compare the results in UC/IPAA patients with those of patients with familial adenomatous polyposis (FAP) with IPAA and UC patients with ileorectal anastomosis (IRA). Five hundred millilitres of a fermented milk product (Cultura) containing live lactobacilli (La-5) and bifidobacteriae (BB-12) was given daily for 4 weeks to 51 UC patients and 10 patients with FAP, operated on with IPAA, and six UC patients operated on for IRA. **No adverse events related to consumption of product were reported.**

U) Gatt, M., et al., *Randomized clinical trial of multimodal optimization of surgical care in patients undergoing major colonic resection.* Br J Surg, 2005. **92**(11): p. 1354-62.
Thirty-nine patients undergoing major elective colonic resection were randomized to receive a ten-point multimodal optimization package or conventional perioperative care. The optimized program included the supplementation of Trevis (Lactobacillus acidophilus La5, Lactobacillus bulgaricus, Bifidobacterium lactis BB-12 and Streptococcus thermophilus) daily 7-14 days before surgery. Twenty-four patients developed complications, nine in the optimized group and 15 in the control group, and five patients required readmission within 30 days of surgery (one and four, respectively). **There was one death, which occurred after a perioperative myocardial infarct in a patient randomized to multimodal optimization. There were no differences in morbidity and mortality between the optimization and conventional group.**

V) Wildt, S., et al., *Probiotic treatment of collagenous colitis: a randomized, double-blind, placebo-controlled trial with Lactobacillus acidophilus and Bifidobacterium animalis subsp. Lactis*. Inflamm Bowel Dis, 2006. 12(5): p. 395-401.
In a randomized, double blind, placebo-controlled trial, 29 patients with collagenous colitis and diarrhea were divided into two groups; 21 patients consumed Lactobacillus acidophilus LA-5 and Bifidobacterium animalis subsp. lactis BB-12 capsules (AB-Cap-10) and 8 patients consumed a placebo for 12 weeks. The overall tolerance of AB-Cap-10 and placebo was good, with only minor adverse events. **In no case were side effects the cause of withdrawal from study.** Only the gastrointestinal symptoms were considered possibly related to AB-Cap-10. In the probiotic group, worsening of diarrhea (n=1), abdominal pain and constipation (n=2), stomach burn (n=1), nausea (n=1), and flatulence (n=1) were reported. In the placebo group, 4 patients complained of nausea.

W) Hol, J., et al., *The acquisition of tolerance toward cow's milk through probiotic supplementation: a randomised, controlled trial*. J. Allergy Cli. Immunol., 2008, **121**(6) : p. 1448-1455.
In a randomized, double-blind, placebo controlled trial, 119 infants between the of ages 1.4 to 6 months with cow's milk allergy were assigned to two groups to receive extensively hydrolyzed formula or formula with *Bifidobacterium lactis* BB-12 and *L. casei* CRL 431 for twelve months. The tolerance to cow's milk was similar at 6 and 12 months. Probiotic intake was confirmed because probiotics were isolated from the feces more often in the treated infants than the control infants. Parents were asked to document whether their infants showed any symptoms as a result of the study formula. **The study formula with or without the probiotic supplementation was well tolerated. They did not note any difference between the groups.**

X) Kajander, et al., *Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota*. Aliment Pharmacol Ther. 2008, **27:** p.48-57.

In a randomized, double-blind, placebo-controlled study, irritable bowel syndrome (IBS) patients were randomized to receive once daily either 1.2dL of a probiotic milk-based drink containing *Bifidobactium lactis* (BB-12), *L. rhamnosus GG* (LGG), *L. rhamnosus Lc705*, and *P. shermanii JS* or 1.2dL of a placebo drink devoid of probiotics but similar otherwise to the probiotic drink. The purpose of the study was to evaluate the probiotic drink compared to the placebo on the health-related quality of life and IBS symptoms of each patient. **At 20 weeks, the IBS symptom score of subjects in the probiotic group decreased significantly, with a 37% reduction compared to baseline,** while the placebo-controlled group had a 9% reduction in IBS symptoms. **Significant beneficial effects were also seen on the quality of life of patients in the probiotic group.**

Y) Meng, et al., *Effect of a lactose-free milk formula supplemented with bifidobacteria and streptococci on the recovery from acute diarrhea*. Asia Pac J Clin Nutr. 2008, **17**(1): p.30-34
In a double-blind, randomized, placebo-controlled study, 212 infants between the ages of 6 and 36 months were studied to determine the effect of a lactose-free formula, supplemented with *S. thermophilus* and *B. Lactis* BB-12 at concentrations of 10 e 8 and 10 e 9 cfu/gram of powder, on the duration of the infants' episodes of acute diarrhea. 71 infants were randomized to the control group, a milk-based lactose-free formula, 71 infants were randomized to receive the same formula but supplemented with 10 e 8 CFU/g BB-12, and 70 infants were randomized to receive 10 e 9 CFU/g BB-12 in their formula. **The duration of the diarrhea episodes was not significantly different among the three groups.**

Z) Smerud, et al., *Effect of a probiotic milk product on gastrointestinal and respiratory infections in children attending day-care*. Microbial Ecology in Health and Disease. 2008, pp 1-6.
In a randomized, double-blind, placebo controlled trial 240 children between the ages of 12-36 months were studied to determine if three probiotic strains (*L. rhamnosus GG*, *L. acidophilus* La-5, *Bifidobactium lactis* BB-12) given daily during their first year of day-care could prevent infections versus a placebo product. The children were asked to drink 150ml of investigational product every day. The product had > 10 e 8 cfu / ml of LGG and BB-12 and > 10 e 7 cfu/ ml of La-5. **The probiotic reduced the number of days with gastrointestinal symptoms (1.7 days for the probiotic product compared to 3.0 days for the placebo, i.e. 43% lower mean of the probiotic product, p=0.02) No significant difference between treatments was seen with respect to respiratory symptoms.**

AA) Rautava, et al., *Specific probiotics in reducing the risk of acute infections in infancy – a randomised, double-blind, placebo-controlled study*. Brit J Nutr. 2009, **101:** p.1722—1726.
In this study, 81 infants before the age of 2 months were randomized to receive formula supplemented with *Bifidobactium lactis* (BB-12) and *L. rhamnosus GG* (LGG) or normal formula daily until the age of 12 months to determine whether probiotics can be effective in reducing the risk of infections in infancy. The study probiotics **reduced the risk of early acute otitis media and need for antibiotic treatment** during the first 7 months of life, and **significantly reduced the incidence** of recurrent respiratory infections during the first 12 months of life. **No serious adverse effects resulting from probiotic supplementation were detected during the study.**

AB) Dotterud, et al., *Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial*. Brit J Dermatol. 2010, **163**: p.616–623.
The goal of this study was to determine whether a probiotic supplement given to pregnant women during the last 4 weeks of pregnancy up until 3 months after birth would reduce the incidence of allergic disease and allergic sensitization in their children at 2 years of age compared with a placebo. The pregnant women were randomized to receive daily either 250mL probiotic low fat fermented milk, containing *L. acidophilus* (La-5), *Bifidobactium lactis* (BB-12), and *L. rhamnosus GG* (LGG) or 250mL placebo

skimmed fermented milk. **The cumulative incidence of atopic disease was significantly reduced in children born to mothers treated with a probiotic supplement for 4 months.** However, there was no reduction in the incidence of asthma or allergic rhinoconjunctivitis in the probiotic group.

AC) Merenstein, et al., *The study to investigate the potential benefits of probiotics in yogurt, a patient-oriented, double-blind, cluster-randomised, placebo-controlled, clinical trial*. Eur J Clin Nutr. 2010, **64:** p. 685-691.
In this study, 182 subjects between the ages of 1-3 years were studied to determine if daily consumption of a probiotic-supplemented yogurt-based beverage containing *Bifidobactium lactis* BB-12 can reduce daycare absences. The children were asked to drink 4 oz. of active or control drink for 90 consecutive days. The active drink had > 10 e 10 cfu / serving of BB-12. **The active drink did not decrease absences due to illness in daycare/school for healthy children. The strain of probiotic studied did not show any positive impact on absences.**

AD) Simren, et al., *Clinical trial: the effects of a fermented milk containing three probiotic bacteria in patients with irritable bowel syndrome—a randomized, double-blind, controlled study*. Aliment Pharmacol Ther. 2010, **31:** p.218-277
This study aimed to assess the effect of milk fermented with *L. bulgaricus* and *St. thermophiles* and containing *L. acidophilus* (La-5), *B. Lactis* BB-12, and *L. paracasei* F19 versus acidified milk without the bacteria on the symptoms of patients with irritable bowel syndrome (IBS). 74 IBS patients were randomized to receive daily 400mL of either the active or control drink for 8 weeks. In both groups, the severity of IBS symptoms was gradually reduced over the 8-week period. **However, a clearly positive effect of probiotic milk could not be detected on the symptoms of IBS patients compared to the control treatment.**

AE) Merenstein et al., *Study to investigate the potential of probiotics in children attending school.* Eur J Clin Nutr. 2011, **65:** p.447-453.
In a randomized, double-blinded, placebo-controlled trial, 172 subjects between the ages of 2-4 years were studied to determine if once-daily consumption of a probiotic-supplemented yogurt-based beverage containing *Bifidobactium lactis* (BB-12) can reduce daycare absences. 91 children were randomized to receive the active drink and 81 children were randomized into the control group. The active drink had > 10 e 10 cfu / serving of BB-12. **The probiotic-containing yogurt-based beverage did not decrease absences due to illness in daycare/school for healthy children.**

AF) Taipale, et al., Bifidobacterium animalis subsp. lactis BB-12 in reducing the risk of infections in infancy. Brit J Nutr. 2011, **105:** p.409-416
In this double-blinded, placebo-controlled study, 109 newborn 1-month-old infants were randomized to receive either a *B.lactis* BB-12 tablet or the control placebo tablet in order to investigate the impact of BB-12 supplementation on the risk of acute infectious diseases. The tablets were administered twice daily until the age of 8 months**. The study demonstrated that BB-12 supplementation significantly reduced the incidence of respiratory infections during the first 8 months of life in healthy breastfed infants.**

AG) Wildt, S., et al., *A randomised double-blind placebo-controlled trial with Lactobacillus acidophilus La-5 and Bifidobacterium animalis subsp. lactis BB-12 for maintenance of remission in ulcerative colitis*. J Crohns Colitis. 2011, **5**: p.115-121.
Thirty-two patients with ulcerative colitis (UC) were randomized to receive either Probio-Tec AB-25, a probiotic capsule containing *L. acidophilus* (La-5) and *Bifidobactium lactis* (BB-12), or a placebo capsule in order to study the clinical effect of probiotic treatment in maintaining remission in patients with UC. The

study drug was administered for 52 weeks. Of the 20 patients randomized to the experimental group, 5 patients (25%) maintained remission after one year of treatment, while only 1 patient out of the 12 in the placebo group maintained remission. **ProbioTec AB-25 demonstrated no effect on maintenance of remission in patients with UC.**

AH) Jacobs, S.E., et al., *Probiotic Effects on Late-onset Sepsis in Very Preterm Infants: A Randomized Controlled Trial*. Pediatrics. 2013, **132**: p.1055-1062.
In this double-blind, placebo-controlled, randomized trial, very preterm infants defined as born <32 weeks' gestation and weighing <1500g were supplemented soon after birth with either probiotics containing *S. thermophilus*, *B. Lactis* BB-12, and *B. infantis* BB-02 or placebo to determine the effect of the daily administration of probiotics on the incidence of definite late-onset sepsis. 1099 infants were enrolled with 548 randomized to the probiotic group and 551 to the control group. The study found **no significant effect** of the probiotic combination on definite late-onset sepsis in very preterm infants.

AI) Smith, T.J., et al., *Effect of Lactobacillus rhamnosus LGG and Bifidobacterium animalis ssp. lactis BB-12 on health-related quality of life in college students affected by upper respiratory infections*. Brit J Nutr. 2013, **109:** 1999—2007.
The aim of this study was to determine the effect of probiotics on the health-related quality of life in college students suffering an upper respiratory infection (URI). In this double-blinded, placebo- controlled trial, 231 healthy students at Framingham State University were randomized to receive a daily dose (5g) of either strawberry-flavored probiotic powder or strawberry-flavored placebo powder. Each powder package contained 10e9 cfu of LGG and BB-12. **The duration of URIs in the probiotic group was significantly shorter than the duration of the URIs of the control group. Also, the probiotic group had URIs of significantly lower severity compared to the placebo group.**

AJ) Fox M, et al., *Can probiotic yogurt prevent diarrhoea in children on antibiotics? A double-blind, randomised, placebo- controlled study.* BMJ Open. 2015; **5**(1): e006474.
In this study, seventy-two children between the ages of 1-12 years prescribed broad-spectrum oral antibiotics were studied to examine the efficacy of yogurt containing *Lactobacillus rhamnosus GG* (LGG), *Bifidobacterium lactis* (Bb-12), and *Lactobacillus acidophilus* (La-5) in reducing the rate of antibiotic-associated diarrhea (AAD). Seventy children, thirty-six of whom received the pasteurized placebo yogurt and thirty-four of whom received the probiotic study yogurt, completed the trial, which consisted of consuming two 100g tubs of yogurt/day for the entire duration of their antibiotic treatment. **The probiotic group had significantly fewer instances of adverse events. Children in the probiotic group had fewer instances of diarrhea and no cases of severe diarrhea, compared to the placebo group.**

APPENDIX 3: SUBSTUDY ADDENDUM A

Background and Objectives:

Antibiotic medicines are often used to fight infectious diseases in humans and prevent bacterial infection. The implementation of antibiotics into medical practice has had an immeasurable positive impact on world health since their discovery in the early 20th century. However, despite the strengths of antibiotic use in medicine, numerous negative aspects have become more apparent in the last 20-30 years. Specifically, antibiotic abuse, which has resulted in the rise of many antibiotic-resistant bacteria such as MRSA and *Clostridium difficile*. These negative aspects of antibiotic overuse and misuse have resulted in many patients experiencing antibiotic-associated diarrhea (AAD) and increased mortality due to infectious diseases [1].

Oral or intravenous antibiotics can significantly alter the composition of gut microbiota, which increases a patient's likelihood of experiencing AAD. Studies have shown that probiotics have the potential to reduce the rate of AAD in patients taking antibiotics, and as a result, many physicians have begun recommending that patients take probiotics supplements when starting a course of antibiotics [2]. Probiotics, which are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [3]. Probiotics not only have the potential to reduce a patient's risk of experiencing AAD, but they also play an essential role in maintaining homeostasis and immunologic equilibrium in the gastrointestinal tract [4,5].

Another critical aspect of maintaining gastrointestinal health is the consumption of prebiotics along with probiotics. Prebiotics are defined as a substrate that is selectively utilized by host microorganisms conferring a health benefit [6]. Prebiotics can be acquired through supplements or fiber-rich foods, which are carbohydrate-based; however, not all fiber is considered a prebiotic [6]. To be considered a prebiotic, the substrate must resist gastric acidity, hydrolysis by mammalian enzymes, absorption in the upper gastrointestinal tract, as well as be fermented by the intestinal microflora and selectively stimulate the growth or activity of intestinal bacteria [7].

The mechanism of prebiotics works primarily through their main fermentation pathway, in which the gut generates pyruvate from hexoses in the undigested carbohydrates. Gut bacteria use a range of carbohydrate hydrolysis enzymes to produce hydrogen, methane, carbon dioxide, short-chain fatty acids (SCFAs), and lactate [7]. Specifically, SCFAs are an important product of this mechanism because of their many significant contributions to human health, including nourishing cells that line the cut, enhancing Ca+ absorption, relieving constipation and diarrhea, as well as entering the bloodstream and traveling to other organs, and in doing so, act as signals to communicate with the brain and regulate the immune system and inflammation [7].

For this substudy, we aim to use the existing data to explore the effect that a participant's dietary fiber intake has on the occurrence of adverse events related to undergoing a 7-day course of antibiotics. No new data will be collected. Additionally, this substudy seeks to examine if higher consumption of dietary fiber impacts the effect of antibiotic-induced reduction in SCFA concentration as reflected by the levels of acetate in stool samples. The primary hypothesis of this substudy is that a higher intake of dietary fiber will result in participants experiencing fewer adverse events from the antibiotic. Further, this substudy's secondary hypothesis is that a higher intake of dietary fiber will result in less of a reduction in SCFAs by day 7, as well as a quicker return to baseline SCFA.

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APPENDIX 4: SUBSTUDY ADDENDUM B

Background and Objectives:

Antibiotic associated diarrhea (AAD) is one of the most common side effects of antibiotic treatment.[1] AAD typically occurs in 5-35% of patients who take antibiotics.[2] Probiotics are live microorganisms that can have a positive effect on the microflora of the gut by restoring microbial imbalance that may have been caused by antibiotics.[3]

Although male and female digestive systems are similar, anatomical and physiological differences cause variances in digestion, including reactions to food, medications, and metabolism. Women have increased sensitivity throughout the whole digestive tract making them more vulnerable to GI pain, bloating, and nausea.[4] On average, the female colon is 10 centimeters longer than the male colon, contributing to a longer average digestive time of females compared to males.[5] A Mayo Clinic Study found that it took males an average of 33 hours to digest food, while it took females an average of 47 hours.[6] A study examining physician visits for constipation noted that females were more likely to report GI discomfort and seek medical assistance when ill.[7]

Given these differences, it is hypothesized that females in the parent studies (R61/YOBIOTIC and R33/OURBIOTIC phases) would report more GI related adverse events since their biological anatomy tends to predispose them to increased GI discomfort.

We observed more incidences of adverse events reported by females compared to males in the current OURBIOTIC study. For this substudy, we propose analyzIng the previously collected data on the adverse events reported by males and females. Since the YOBIOTIC study is so similar to the OURBIOTIC study, we propose studying both cohorts to increase the sample size. Another aim would be to compare any differences between males and females in the Short Chain Fatty Acid data.

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