Efficacy and Safety of BB-12 Supplemented Strawberry Yogurt For Healthy Children on Antibiotics (IND # 13691)

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1 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.

2 PROTOCOL SUMMARY

Title:	Efficacy and safety of BB-12 supplemented strawberry yogurt for healthy children on antibiotics
Phase:	Phase 2
Population:	300 Healthy Children on Antibiotics for a Respiratory Infection
Number of Sites:	Single Center
Study Duration:	60 months
Subject Participation Duration:	7-10 days of active intervention, follow-up until Day 21, and one final follow-up on Day 180
Description of Agent or Intervention:	Probiotic, BB-12, supplemented yogurt, 4 ounces taken orally for 7-10 days, concurrently with 7-10 course of antibiotics
Objectives:	**Primary Objective:** To test the efficacy of *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strain BB-12 (BB-12) in preventing diarrhea compared to a control in children taking antibiotics. **Secondary Objective:** To assess the safety of strawberry flavored yogurt supplemented with BB-12.
Description of Study Design:	Randomized, double-blinded, controlled clinical trial
National Clinical Trial (NCT) Number:	NCT03181516
Georgetown University Institutional Review Number:	2016-1489

3 KEY ROLES

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4 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

4.1 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.

4.1.1 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]

4.1.2 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]

4.1.3 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.

4.1.4 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.

4.1.5 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."

Summary of BB-12 Safety Articles, with the identical strain we will be using:

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 Pediatr Gastroenterol 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 was 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 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, ten 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, ten 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. 211-217.

In a double-blind, randomized, controlled study, 16 patients with restorative proctocolectomy for ulcerative colitis randomized to two groups: eight 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. Both products were consumed 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 *St. 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. Probiotics also 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, only 5 patients (25%) maintained**

remission after one year of treatment, while only 1 patient out of the 12 in the placebo group maintained remission. **Therefore, the researchers could not demonstrate that ProbioTec AB-25 had an 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 *St. 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 college 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 10 e 9 cfu of LGG and BB-12. **The duration of URI's in the probiotic group was significantly shorter than the duration of the URI's of the control group. Also, the probiotic group had URI's 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 the 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.**

4.1.6 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]

4.1.7 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.

4.1.8 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.

4.1.9 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

SIPPY I Events	**Control**	**BB-12**
	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	**Control**	**BB-12**
	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

4.2 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.

More specifically, the rationale for this Phase I study is to determine safety of our drink and comply with the FDA's recommendations pertaining to an IND application, we will conduct a phase I safety study.

4.3 Potential Risks and Benefits

4.3.1 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.

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 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.

4.3.2 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 varied diseases including diarrhea, asthma, necrotizing enterocolitis and allergies. It has been also found to significantly improve skin conditions in eczema patients.

Yogurt: Yogurt has its own known benefits coming from milk. For every 6-ounce of 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 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.

5 OBJECTIVES/STATISTICAL METHODS

5.1 Study Objectives

5.1.1 Primary Objective

Objective 1: To test the efficacy of high dose BB-12 supplemented yogurt in preventing AAD, compared to yogurt without BB-12, in children receiving antibiotics.

5.1.2 Secondary Objectives

Objective 2: To assess the safety of yogurt supplemented with BB-12.

Objective 3: To carry out longitudinal community structure and gene expression analysis of fecal microbiota to evaluate the impact of high dose BB-12 in a pediatric population receiving antibiotics.

5.2 Study Outcome Measures

5.2.1 Primary Outcome Measure

The primary outcome is clinically diagnosed diarrhea anytime within 14 days of enrollment. Diarrhea is clinically defined as three or more loose stools per day for two consecutive days. This will be a dichotomous (yes or no) outcome of diarrhea during the 14-day period.

5.2.2 Secondary Outcome Measures

We will be evaluating safety as per FDA regulations on adverse event collection. Parents/caregivers (older children, themselves) will keep a daily diary to track: bowel movements via Bristol scale (see **Appendix**),[103] drink compliance, and adverse events. Secondary outcomes include: duration of diarrhea, respiratory illness duration, a validated pediatric quality of life score for all participants over age 2,[104, 105] stool frequency, stool form measured by Bristol scale,[103] treatment failure (defined as a return to the doctor's office with worsening symptoms), antibiotic compliance, missed school, and missed activities.

6 STUDY DESIGN

This is a randomized, single center, Phase 2 study to test the efficacy of *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strain BB-12 (BB-12) yogurt in preventing AAD, compared to yogurt without BB-12, in children receiving antibiotics for a respiratory infection. Three hundred participants will be randomized to two groups, BB-12 supplemented yogurt and control yogurt, using the random allocation rule. This randomization approach will ensure equal allocation of subjects to each group. Participants will be recruited through Capital Area Primary Care Research Network (CAPRICORN), a practice-based research network (PBRN). Children prescribed a 7-10-day penicillin or cephalosporin class antibiotic for a respiratory infection, who meet all the inclusion and exclusion criteria will be enrolled. There will be no exclusion based on race, gender, or insurance status. Based on our previous experience with clinical trials, we will be able to enroll 75 participants in the first year.

7 PARTICIPANTS

7.1 RECRUITMENT STRATEGY

Flyers and posters will also be displayed in all offices participating in the study prior to initiation of the study.

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 by the board of directors and sent out to physicians for comment. Thus, not all physicians or offices participate in each study; but are allowed to choose studies that they have interest in and works with 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.

7.2 INCLUSION CRITERIA

We will enroll 300 participants, half for the BB-12 yogurt drink and half for the control yogurt. All parents of children 3-12 prescribed a penicillin or cephalosporin class antibiotic for a respiratory infection will be alerted of the study by their physician or by the study team.

Inclusion: Participants will be *eligible* if:

1. Child is between ages of 3-12
2. Caregiver has the ability to read, speak and write English or Spanish
3. Household has refrigerator for proper storage of drink
4. Household has telephone access
5. Enrollment must take place within 24 hours of starting antibiotics
6. Outpatient treated
7. Treatment with a penicillin or cephalosporin class antibiotic regimen for 7-10 days for a respiratory infection; the following is a list of inclusive antibiotics:
    a) Amoxicillin
    b) Augmentin (amoxicillin/clavulanate)
    c) Ancef (cefazolin)
    d) Cefadroxil
    e) Cephalexin
    f) Cephradine
    g) Duricef (cefadroxil)
    h) Keflex (cephalexin)
    i) Kefzol (cefazolin)
    j) Velosef (cephradine)
    k) Ceclor (cefaclor)
    l) Cefotan
    m) Cefoxitin
    n) Ceftin (cefuroxime)
    o) Cefzil (cefprozil)
    p) Lorabid (loracarbef)
    q) Mefoxin (Cefoxitin)
    r) Zinacef (cefuroxime)
    s) Omnicef (cefdinir)
    t) Suprax (cefixime)
    u) Dicloxacillin
    v) Pen-Vee K (penicillin)

A respiratory infection will be classified as any infection the physician designates as strep or non-strep pharyngitis, otitis media, pneumonia, sinusitis or bronchitis that results in a prescription of antibiotics.

7.3 EXCLUSION CRITERIA

The exclusion criteria are:

1. Developmental delays
2. Any chronic condition, such as diabetes or asthma, that requires medication
3. Prematurity, or born prior to 37 weeks gestation/of pregnancy
4. Congenital anomalies
5. Failure to thrive
6. Allergy to strawberry
7. Active diarrhea (diarrhea is defined in this study as three or more loose stools per day, for two consecutive days)
8. Any other medicines used except anti-pyretic medicines (*pro re nata* concomitant medications are allowed)
9. Parental belief of lactose intolerance
10. History of heart disease, including valvulopathies or cardiac surgery, any implantable device or prosthetic
11. History of gastrointestinal surgery or disease
12. Milk-protein allergy
13. Allergy to any component of the product or the yogurt vehicle
14. Allergy or a hypersensitivity to the antibiotic prescribed by her/his provider
15. Allergy to any of the following medications:
    a) Tetracycline
    b) Erythromycin
    c) Trimethoprim
    d) Ciprofloxacin
16. Blood oxygen saturation <90% (if enrollment/baseline visit is completed in person and if the participant was prescribed antibiotics during a telemedicine visit)

Participants will also be asked to refrain from any probiotic foods or supplements during the entire 2 weeks of study and will be supplied with a list of these products.

7.4 RANDOMIZATION

The randomization scheme will be generated using permuted blocks of varying size. 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 active group receives.

7.5 INFORMED CONSENT AND ASSENT

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 parents. Consent forms describing in detail the study interventions/products, study procedures, and risks are given to the parents 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 parents to ensure that they understand the nature of the research and can voluntarily decide whether or not to participate.

The assent process is similar to the informed consent process for adults. The research team will discuss the purpose of this research project, procedures involved, potential risks and benefits, how we will protect their personal information, compensation, freedom to withdraw (change their mind), participant responsibilities and permission to participate. The assent and informed consent processes will allow the child and parent to ask any questions and decide amongst themselves whether or not to participate. The language of the assent process will be adjusted to be age and maturity level appropriate. Assent must be affirmative. The parent or child may discontinue study participation at any time. The assent and documentation process will be as follows:

For children under the age of 7:
Only parental permission is required (signed Informed Consent form containing the required elements of consent) before study-related procedures are conducted. In some cases (e.g. children ages 5-6), the researcher may determine the child is capable of being included in the assent process. While assent will not be required, in these cases, a simple oral explanation of the study will be offered to the child. There will be no separate form but the process will be documented in the participant's study file.

For children ages 7-12 years old:
A more complete oral description of the research is given to the child, along with a one-page written simplified summary of the study (such as the elements on the Informed Consent Form). An Assent of the Child addendum to the Informed Consent Form must be completed, along with the signed parental Informed Consent form. The child's signature is not required but the assent process will be documented in the participant's study file. The Assent of the Child addendum will specify if the child chose to sign the form or if it was determined that the child did not have the capability to give assent.

In the situation where participants and their parents/guardians elect to or must sign electronically, the following will occur:
Research personnel will review the informed consent (IC), research authorization (HIPAA), and (where applicable) assent forms with a participant and their parent(s)/guardian(s), and the research team will obtain signatures via the Georgetown-licensed version of DocuSign (HIPAA compliant).

- Potential participants and their parents/guardians would be instructed to use personal (rather than public) electronic devices (computers, tablets, phones, etc.) in signing with DocuSign.
- Parents/guardians—and participants in the case of assent—would be asked to specify an email address at which they can access and sign IC, HIPAA, and assent using DocuSign.
- Once signed by the parent/guardian, electronic versions of IC and assent (where applicable) would be signed by the research staff member who obtained consent and returned via email to the parent/guardian.

7.6 PARTICIPANT STIPENDS

Participants and parents/caregivers of participants will receive compensation for their time. Compensation will be given to the parents/caregivers five times throughout the study. All stool 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.

8 INVESTIGATIONAL PRODUCT

8.1 STUDY PRODUCT DESCRIPTION

8.1.1 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 yogurt beverage.

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

8.1.2 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 quart-size plastic containers, which will yield 8 servings of the 4-ounce daily dosage. Two of these containers of yogurt drink will be provided to the participants at enrollment 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.

8.1.3 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 describe 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 SN2000036366	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.

8.1.4 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. [104] 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^{TM}, Burlington, North Carolina) and Petrifilm^{TM} aerobic plate counts according to the 17th edition of Standard Methods for the Examination of Dairy Products, #7.072 and #6.040, respectively.

8.1.5 PCR Verification of BB-12 Level in Finished Drink

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. [105] Cells are lysed according to the microwave method of Bollet et al. as modified by Kullen et al. [106, 107] 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.

8.1.6 Acceptability Testing

Pilot testing of the drink was carried out among fifteen children ages 2-7. All children were able to consume the drink without any complaints and 13 out of the 15 requested more after consuming approximately 3 ounces of the drink.

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.[106, 107]

8.1.7 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

8.2 Dosage, Preparation and Administration of Investigational Product

Enrolled participants will be given a total 7-10-day supply of the yogurt (an amount sufficient to be taken concurrently with a 7-10 course of antibiotics) with instructions to the parents/caregivers on how to administer the product. Participants will drink four (4) ounces of yogurt each day for ten consecutive days. Yogurt will be administered via mouth.

8.3 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.

8.4 Product Transportation, Handling and Storage

8.4.1 While the Product is being Shipped from the Penn State Area to the Washington, DC Area

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.

8.4.2 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—one for Drink # 1-3 and one for Drink # 4-6. 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. Once a month, the study personnel will make arrangements with Georgetown University Facilities Management to dispose of the products before or on the date of expiration.

8.4.3 Transportation and Storage While being Dispensed to Participants

The products will be transported to the participants' homes in coolers packed with ice and equipped with a VWR® Digital Refrigerator/Freezer Thermometer with Alarm. Prior to each delivery, the study personnel will arrange and confirm a mutually convenient time with the parents/caregivers to ensure that they will be home to receive the drink. This ensures that the parents/caregivers can place the yogurt drinks into their home refrigerators immediately upon receipt.

8.4.4 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 parents/caregivers due to spillage, spoilage or any other reasons as needed.

8.4.5 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 7-10-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. Parents/caregivers 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.

8.5 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.

Collection: Stool samples from the child will be collected by the parents/caregivers 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 9.5, "Stool Collection Instructions for Participants.")

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 -40^0 Celsius to freeze for batch transport to the Institute for Genomic Sciences at the University of Maryland Baltimore (Fraser Lab). (Refer to Section 9.4, "Stool Collection Instructions for Study Personnel.")

Analysis: Stool 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 min heating step at 95°C. The isolated DNA samples are subjected to *B. lactis* specific PCR analysis (See procedure in 8.1.5 PCR Verification of BB-12 Enumeration). A portion of the stool collected at each sampling point will be held at -70ºC for possible future investigation.

In addition to stool analysis, we will ask parents to record how much yogurt their child consumed on a daily basis.

9 STUDY SCHEDULE

9.1 RECRUITMENT

Research staff will meet with area physicians via the CAPRICORN network and inform them about the study. All treating physicians at each office will alert patients who are being placed on an antibiotic for a respiratory infection that a research study examining the safety of a probiotic yogurt drink is being carried out by Georgetown University researchers. Flyers and posters will also be displayed in all offices participating in the study three months prior to initiation of the study.

If they meet basic entry criteria, the potential participant will be offered the opportunity for study participation and consent will be obtained. Information provided during the consent process include, the purpose of the study, procedures, withdrawal procedures, subject termination, risk/discomforts, benefits, costs, compensation, and alternative to participation.

9.2 ENROLLMENT/BASELINE

The enrollment/baseline visit will take place at the participants' homes or doctor's office where comfort and confidentiality is assured. 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.

The enrollment/baseline visit will be completed virtually, when public health guidelines recommend limiting in-person visits or when participants prefer a virtual visit.

Once it is confirmed that the parents/caregivers and child meets all inclusion and exclusion criteria, baseline data will be collected. These include: Baseline Health Status and Demographic Information.

Following the baseline procedures, the parents/caregivers will receive the BB-12 yogurt or control yogurt for days 1 through 7-10 to be given to their child once a day. In addition, they will also receive measuring cups that denote 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.

Parents/caregivers will be asked to collect a stool sample from their child prior to starting the yogurt. Stool for children who stool in a toilet will be collected via a stool hat and transferred to proper vials. Stools for children in diapers will be collected directly from the diapers. 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, parents/caregivers will also be given all materials to collect stools, the diary, and study materials as well as the initial compensation.

Following virtual enrollment/baseline visits, study supplies typically delivered at enrollment—prescribed investigational product (yogurt), forms for data collection, and kits for sample collection—will be delivered following the informed consent process and after all required signatures have been obtained.

9.3 TELEPHONE FOLLOW-UP

On the first day that the participant drinks the yogurt, parents will schedule a phone call for 30 minutes after the drink is consumed, to ensure any adverse events are recorded. Parents will alert study personnel what time their child will consume the first yogurt and study personnel will call the participant 30 minutes after to ensure that there are no side effects from the yogurt.

These follow-up phone visits will be completed on Days 7, 11, 14 and 180 (±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 parent/caregiver'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 parent/caregiver'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 in during the study, 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.

Additional stool samples will be collected on Days 7, 14, 21 and 30 (±2 days) (**Table 8**).

9.3.1 Telephone Follow Up Script

In general, the Day 7, 11, 14, and 180 (±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 Number:* Make sure the parent/caregiver has the correct drink number as you are completing the Follow-Up Form.

    a) Ask the parent/caregiver the number on the drink bottles and this should be confirmed with the computer program provided by the data manager.

    b) If this number is not consistent, the parent/caregiver should be alerted not to give their child any more drinks and the project coordinator should be immediately alerted after completing this week's Follow-Up Form

2) *Daily Assessment Diary:* Ask the parent/caregiver 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 parent/caregiver 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 parent/caregiver 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 daycare, etc., this should be generally whole integers. However, it is possible that a child 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 parent/caregiver 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 your child visit a doctor?" A doctor is defined as any medical personnel in this study. Therefore, the parent/caregiver may say, "Never, but s/he saw a nurse practitioner." Please count this the same as seeing the doctor.

- *Satisfaction Questions:* Please provide the parent/caregiver with the numbers s/he stated the prior time for a comparison.

- *Compliance:* Please let the parent/caregiver know that we would like them to give their child the drink every day, but regardless, we appreciate their participation.

- *Adverse Events:* If the parent/caregiver 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.

5) Confirm the date and time for the next visit's call.

All interventions will be completed by week two of the trial. At day 180, participants will be queried for changes in baseline health, onset of new chronic medical conditions and non-routine medical visits in the past 6 months.

Table 8. Participant Timeline and Sample of Data Collection Schedule (N=300)

Study Day	0/1	2	7	10	11	14	21	30	180[3]
Enrollment (baseline health and demographics)	X								
Antibiotics	|→	→	→ **or** →|	→|					
Yogurt Interventions	|→	→	→	→|					
Fecal Sample Collection	X		X			X	X	X	
Follow-up Data Collection			X		X	X			X
Daily Assessment Diary[1]	|→	→	→	→	→	→|			
16S rRNA Gene Sequences	X		X			X	X	X	
Metatranscriptome Analysis[2]	X		X						
PedsQL™	X		X			X			
Diet Recall Diary (2 x 2 days)		|→	→	→	→	→|			
Clostridium difficile Analysis[2]		|→	→	→	→	→|			
Adverse Event (AE) Reporting	|→	→	→	→	→	→	→	→	→|

[1]Participants keep a diary from Days 1 through 14 (collected on Days 7 and 15). Information on stool number, shape and consistency, health, use of interventions, symptomatic care, quality of life, and AEs.
[2]Will be conducted on all participants with clinically-defined diarrhea.
[3]Day 180 follow-up is required by the FDA for a safety measurement. On day 180 parents will be asked if they believe there were any additional AEs associated with the yogurt intervention that they have not reported during the previous data collection periods.

9.4 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 **Day 1, 7, 14, 21 and 30** 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
- Make sure all caps are tightly closed when collecting sample
- Use the cooler/coolly packs when transporting sample from participant's house to Georgetown University.
- Fill out **Sample Collection Form**
- Before placing the sample in the freezer, make sure:
  1. Any names on the samples are crossed out
  2. Write correct Study IDs

9.5 STOOL COLLECTION INSTRUCTIONS FOR PARTICIPANTS

Stool is to be collected from your child on **Days 1, 7, 14, 21 and 30**.

At enrollment and throughout the study, you will be provided with stool collection materials. This kit will include: disposable gloves, 3 stool containers/vials, small plastic zipper bags, 1 large plastic zipper bag, labels and a stool collection device (a "stool hat" – if your child is toilet trained). 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. You will be provided with labels. Please write your child's **STUDY ID** number (if not already on the label) and the date and time the stool was collected. Do this on the label of the 3 stool containers (vials) and once on the label on the plastic zipper bag. **Please do not write your child's name on the labels**.

4. **If your child is toilet trained**: Lift the toilet seat. Align the "stool hat" with the rear of the toilet rim (see Photo A). Replace the seat. Have your child use the toilet as s/he normally would, letting the stool collect in the "hat".

   ***Skip previous step if your child uses diapers**: collect stool directly from diaper.

5. Unscrew the cap from one of the two smaller vials that contains liquid. Use the spoon attached at the end of the cap of vial to fill the container to the fill line indicated on the vial (but do not overfill; see Photo B).

6. Screw the cap back onto the container. 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.

7. For the last container (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 container.

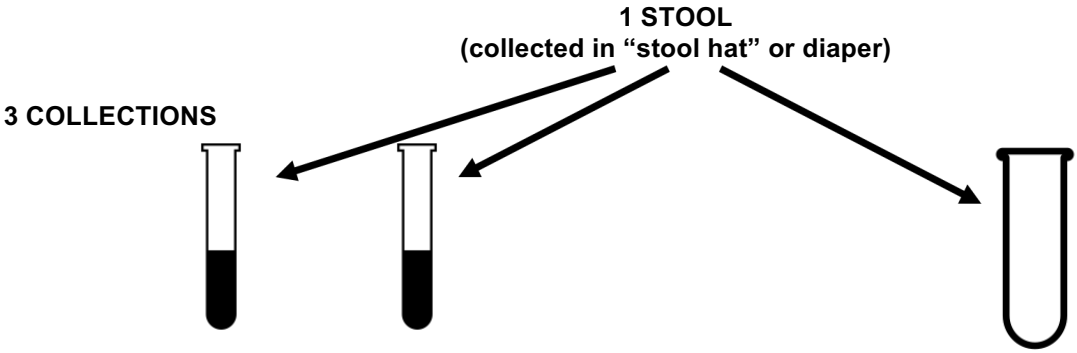
8. Discard remaining stool into the toilet and flush. Discard diaper or stool hat.

9. Place the containers/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.

10. Place the sealed plastic zipper bag into the **FREEZER immediately** for storage.

11. Discard gloves and wash your hands.

12. Contact the research personnel as soon as possible to arrange a time for stool collection pickup, which should typically occur within 24-48 hours.



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

Photo A

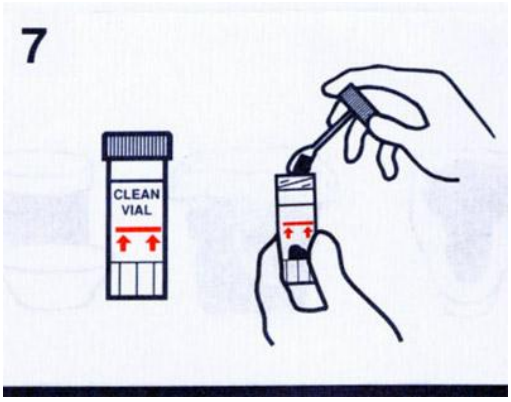


Photo B

10 ASSESSMENT OF SAFETY

We will protect participants by alerting the parents/caregivers 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 parents/caregivers 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 child 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.

10.1 ADVERSE EVENTS

An adverse event (AE) refers to any untoward medical occurrence in a clinical investigation subject administered an investigational product and which does not necessarily have a causal relationship with the treatment. An AE can therefore be any unfavorable and unintended sign, symptom, or disease temporally associated with the use of the investigational product, whether or not considered related to the product. This includes events not present at the start of the study or worsening during the course of the study even if present at the study start. An AE can be reported anytime after study enrollment; a participant is considered enrolled once the informed consent form is signed by the parent/caregiver.

Details of all solicited and unsolicited adverse events reported by parents/caregivers or observed by research personnel must be recorded in the adverse event report forms. Parents/caregivers will be provided with cards, which will list the most likely possible adverse events (see **Table 9**), where they can record solicited and other unsolicited events not already listed. All adverse events observed must be recorded separately and all must be reported to the IRB on IRB AE forms. All AEs regardless of severity level will be evaluated by full DSMB during scheduled meetings. The severity of adverse events will be recorded as follows:

- Mild (Grade 1): no or minimal interference with the subject's daily activity with intervention not indicated
- Moderate (Grade 2): some interference with the subject's daily activity but with intervention indicated
- Severe (Grade 3): prevents subject's daily activity and requires medical intervention
- Potentially Life Threatening (Grade 4): ER visit or hospitalization, with intervention indicated to prevent permanent impairment, persistent disability, or death
- Fatal (Grade 5): death

Based on the safety record of the yogurt, we anticipate that the following are the most likely AEs:

- GI distress including: bloating, flatulence, nausea, emesis, anorexia, constipation and diarrhea

- Allergic reactions including: anaphylaxis, hives, shortness of breath, rash, wheezing, and other reactions that the parent/caregiver or doctor believes may be related to food.

Potential symptoms relating to the existing respiratory infection (enrollment criteria/indication for antibiotics) may include: cough, earache, nasal congestion, runny nose, or sore throat. Worsening (increase in grade) or the onset of these symptoms not present at the start of the study will be reported as adverse events.

Table 9. Possible expected adverse events and grading scale.[a]

Possible Adverse Event	Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)	Potentially Life Threatening (Grade 4)
Loose stools	Mild symptoms causing no or minimal interference with usual social & functional activities with intervention not indicated	Moderate symptoms causing greater than minimal interference with usual social & functional activities with intervention indicated	Severe symptoms causing inability to perform usual social & functional activities with intervention or hospitalization indicated	Potentially life-threatening symptoms causing inability to perform basic self-care functions with intervention indicated to prevent permanent impairment, persistent disability, or death
Diarrhea (≥3 loose stools per day for 2 consecutive days)	Transient or intermittent episodes of unformed stools OR Increase of 3 stools over baseline per 24-hour period	Persistent episodes of unformed to watery stools OR Increase of 4 to 6 stools over baseline per 24-hour period	Increase of ≥ 7 stools per 24-hour period OR IV fluid replacement indicated	Life-threatening consequences (e.g., hypotensive shock)
Constipation	NA	Persistent constipation requiring regular use of dietary modifications, laxatives, or enemas	Obstipation with manual evacuation indicated	Life-threatening consequences (e.g., obstruction)
Fever Non-axillary (taken by armpit) temperatures only	38.0 to < 38.6°C or 100.4 to < 101.5°F	≥ 38.6 to < 39.3°C or ≥ 101.5 to < 102.7°F	≥39.3 to <40.0°C or ≥102.7 to <104.0°F	≥ 40.0°C or ≥ 104.0°F
Flatulence	Mild symptoms causing no or minimal interference with usual social & functional activities with intervention not indicated	Moderate symptoms causing greater than minimal interference with usual social & functional activities with intervention indicated	Severe symptoms causing inability to perform usual social & functional activities with intervention or hospitalization indicated	Potentially life-threatening symptoms causing inability to perform basic self-care functions with intervention indicated to prevent permanent impairment, persistent disability, or death
Lack of appetite	Mild symptoms causing no or minimal interference with usual social & functional activities with intervention not indicated	Moderate symptoms causing greater than minimal interference with usual social & functional activities with intervention indicated	Severe symptoms causing inability to perform usual social & functional activities with intervention or hospitalization indicated	Potentially life-threatening symptoms causing inability to perform basic self-care functions with intervention indicated to prevent permanent impairment, persistent disability, or death
Pain	Pain causing no or minimal interference with usual social & functional activities[b]	Pain causing greater than minimal interference with usual social & functional activities	Pain causing inability to perform usual social or functional activities	Disabling pain causing inability to perform basic self-care functions[c] OR hospitalization indicated
Rash (Cutaneous reaction)	Localized rash	Diffuse rash OR Target lesions	Diffuse rash AND Vesicles or limited number of bullae or	Extensive or generalized

			superficial ulcerations of mucous membrane limited to one site	bullous lesions OR Ulceration of mucous membrane involving two or more distinct mucosal sites OR Stevens Johnson syndrome OR Toxic epidermal necrolysis
Vomiting	Transient or intermittent AND No or minimal interference with oral intake	Frequent episodes with no or mild dehydration	Persistent vomiting resulting in orthostatic hypotension OR Aggressive rehydration indicated (e.g., IV fluids)	Life-threatening consequences (e.g., hypotensive shock)
Cough Earache Nasal congestion Runny nose Sore throat	Mild symptoms causing no or minimal interference with usual social & functional activities with intervention not indicated	Moderate symptoms causing greater than minimal interference with usual social & functional activities with intervention indicated	Severe symptoms causing inability to perform usual social & functional activities with intervention or hospitalization indicated	Potentially life-threatening symptoms causing inability to perform basic self-care functions with intervention indicated to prevent permanent impairment, persistent disability, or death

[a] U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Division of AIDS. Division of AIDS (DAIDS) Table for Grading the Severity of Adult and Pediatric Adverse Events, Version 2.1. [March 2017].
Available from: https://rsc.tech-res.com/docs/default-source/safety/daids-ae-grading-table-mar2017.pdf
[b] Usual Social & Functional Activities – Young Children: Activities that are age and culturally appropriate (e.g., social interactions, play activities, learning tasks, etc.).
[c] Basic Self-care Functions – Young Children: Activities that are age and culturally appropriate (e.g., feeding self with culturally appropriate eating implement).

If any Serious AEs (SAEs) --defined as any adverse event that results in death, is life-threatening for the person involved, is likely to result in disability or permanent invalidity, results in hospitalization of a subject or prolonged hospitalization (typically Grade 3 or above – see Section 10.1.1) occur, the PI will be alerted immediately as will the IRB. If necessary, members of DSMB will be alerted immediately. The PI and/or IRB will decide if **stopping criteria** have been met. If they believe so, a teleconference will be arranged by the DSMB Administrator. The PI and Data Coordinating Center will be alerted and no more participants will be enrolled until after the meeting, which will occur within 24 hours.

10.1.1 Serious Adverse Events

SAEs might not be related to the drink administration but are still required to be evaluated as above by the DSMB.
An SAE is any adverse event that:

- Results in death
- Is life-threatening for the person involved
- Is likely to result in disability or permanent invalidity
- Results in inpatient hospitalization or prolongation of existing hospitalization of a subject
- Results in a congenital anomaly or birth defect
- May, based on medical judgement, jeopardize the subject and may require medical intervention to prevent on the outcomes previously listed

All AEs will be monitored by the investigator until a satisfactory resolution. 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 all results of examinations and treatment carried out during follow up of SAEs. Additionally, all reports will be provided to the FDA and the NICHD Program Official.

10.1.2 Reporting Guidelines

If a parent/caregiver reports an AE/SAE that does not yet have a Date of Resolution and is continuous (no more than two days without interruption) across two follow-up periods, this AE/SAE will be considered one event. If more than two days pass between the reporting of symptoms, it will be considered as separate events. The total duration of the AE/SAE will be collected but only one AE/SAE Form will be completed for this particular event.

The following applies only for non-serious (typically below Grade 3) events; AEs of Grade 3 or above will follow the aforementioned policies for reporting and monitoring of serious adverse events.
For non-serious events, 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 parent/caregiver or consider Day 14 as the final Date of Resolution:

- If the AE reported is listed on the Adverse Event Report Form (Codes 1-14) as a possible, expected AE, the research assistant will continue to follow-up the parent/caregiver 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 15), or unexpected, and the Principal Investigator AND parent/caregiver 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.

10.2 STOPPING CRITERIA

Drink administration may be suspended if:

- Any study participant experiences death that the DSMB believes may be related to the drink administration.
- If statistically or clinically significant amount of hospitalizations occur that the DSMB believes may be related to drink administration.
- 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.
- Any serious adverse event for which a relationship with the study product cannot be ruled out.
- The occurrence of statistically or clinically significant, similar Grade 2/moderate adverse events in the study population that the DSMB or PI believe is related to the intervention.
- A statistically or clinically significant number of severe adverse events occur in the study population.

Individuals may be withdrawn from the study for any of the following:

- Adverse events; the PI will discuss AEs with the parent/caregiver, and possibly with the participant's physician, as needed, to determine whether the participant should be withdrawn from the study.
- If parents/caregivers or participants are deemed to be non-cooperative (e.g. refuse to collect stools, participate in follow-up calls or fail to complete diaries).
- If parent/caregiver is lost to follow-up, i.e. more than 1 week without contact with study personnel during the follow-up period.
- Serious adverse events related to drink administration, as determined by PI, parent/caregiver or participant's primary care physician.

10.3 Safety Oversight

The Data Safety Monitoring Board, DSMB, will function as an independent oversight committee charged with overseeing participant safety and providing recommendations to the PI and Georgetown University regarding the continuing conduct of the study. The DSMB will review periodic blinded Interim Safety Reports. Additionally, unblinded but sealed Interim Safety Reports will be provided to each voting DSMB member should the committee determine that review of unblinded safety data is warranted.

The DSMB is responsible for monitoring the tolerability, safety and effectiveness of the yogurt. They will meet periodically once the first participant is enrolled and continue to do so as long as participants are actively involved in the study. One meeting will occur before enrollment to review study objectives, data collection and safety measures. Meetings will occur via teleconferences or at Georgetown University.

11 STATISTICAL AND LABORATORY ANALYSES

11.1 STUDY HYPOTHESES

Hypothesis 1: Children receiving antibiotics who receive the yogurt with BB-12 will demonstrate less diarrhea than those receiving a control yogurt without BB-12.

Hypothesis 2: Yogurt containing BB-12 will be safe and well tolerated in this larger pediatric population. This is a Phase II trial that requires additional testing to further evaluate the safety of high dose BB-12.

11.2 SAMPLE SIZE CONSIDERATIONS

Sample Size Calculation for the Primary Outcome: This study is a double blinded, randomized Phase II trial where subjects will be randomly assigned (1:1) to either a probiotic (BB-12) yogurt group or to a control yogurt group. The primary outcome is dichotomous, clinically diagnosed diarrhea or no diarrhea, with the rate of AAD in the general population estimated to be 20-35%. Preliminary results from the Cochrane review showed the incidence of AAD in a high dose probiotic group to be 8% versus 22% in a control group.[108] Using the Cochrane estimates, a total sample of 300 subject (150 per group) would provide a power of 0.92 for detecting a difference of 0.14 (0.22 control - 0.08 probiotic) at alpha = 0.05, 2-tailed. To allow for one interim analysis (at approximately 50% recruitment), and then adjusting the final p-value to 0.029, the power would still be 90% for detecting the difference of 0.14 in the rates of AAD. If the difference in the final rates is smaller than 0.14, such as 0.12, the resulting power would still be sufficiently large (80%) and power would be equal to 74% if the final difference in rates were equal to 0.10. While the hypothesis is one-sided (see Aim 1; we expect children receiving probiotic intervention will demonstrate less AAD than those receiving the control product), we still used a two-tailed alternative for estimating the sample size in order to be conservative. While multiple efforts are arranged to minimize loss of data, in order to obtain the primary outcome (diarrhea or no diarrhea), there still will be some participants lost to follow-up and the endpoint is unknown. Nonetheless, if the lost to follow-up rate is as high as 10% (then N=270, approximately 135 per group), power would still be 90% for detecting the difference of 0.14 and 80% for detecting a difference of 0.12.

11.3 STATISTICAL ANALYSIS PLAN FOR PRIMARY AND SECONDARY OUTCOMES

Initially, descriptive statistics (means, medians, frequency distributions, standard deviations, boxplots, etc.) for all characteristics will be examined for each group. Either parametric or nonparametric statistical (depending on results from the descriptive statistics) tests will be used for comparing results of the randomization. Because recruitment will take place over a long period (projected 3.5 years), descriptive statistics will further be examined in 4-6 month intervals (depending on the frequency of enrollments). This is to check that there are no patterns or differences in the subject characteristics over the enrollment time period. As the trial progresses, these characteristics will be continuously monitored and compared, so at that moment if there are differences in the distributions over time, the study team and the Data Safety Monitoring Board can convene to discuss. Additionally potential covariates, such as, age at entry, sex, weight of subject, time of year, etc., will also be statistically examined using univariate statistical tests.

For testing the primary objective (Aim 1), i.e., comparing the rates of AAD between the BB-12 and control groups, Fisher's Exact Probability test will be used. The relative risk of AAD for BB-

12 versus control and the 95% confidence interval will be calculated to display magnitude of the relationship. If the rates of AAD need to be adjusted for covariates, then multiple logistic regression models will be employed for predicting the adjusted rates of AAD. Covariates will be examined for multi-collinearity so as not to saturate the multiple logistic regression models constructed. With this large number of subjects enrolled, it is not expected that the number of AAD cases will be small enough to warrant further statistical methods. However, if the number of AAD cases is very small then exact statistical testing methods will be exclusively used for making the comparisons and performing any pertinent subgroup analyses. The Pediatric Quality of Life Inventory™ (PedsQL™) has a validated and easy scoring system.[104, 105]

For Aim 2, numerous exploratory techniques (i.e., all descriptive statistics previously mentioned) will be performed to verify that the probiotic is safe and well tolerated. Additionally, the AEs will be collected and studied over time of enrollment. The rates of AEs will be estimated, 95% confidence interval be constructed for describing precision and statistically compared between groups. This will be accomplished using parametric or nonparametric tests. Further, in order to accurately identify patient populations or subgroups that can benefit from the BB-12 probiotic, decision tree models will be examined. Using decision trees, we may be able to identify groups of subjects that have no AEs versus those with an AE. Similarly, cluster analyses may also be employed to identify potential pockets of subjects that share similar characteristics but differ with respect to AEs. This is related to an area known as “unsupervised” modeling in data mining, but is appropriate in this Phase II trial for finding subgroups of subjects that have plausible similarities and also differences.

Statistical analysis 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. One interim analysis will be performed when approximately 50% of the subjects have been recruited. While the alpha for stopping the trial will be set at 0.01, it is unlikely that we would want to stop this trial at that point even if significance is achieved, but the Data Safety Monitoring Board, PI and NIH representative will make that final decision. For Aim 1, the level of significance for the primary objective will be tested at alpha = 0.029 to account for the one interim analysis.

11.4 Longitudinal Community Structure and Gene Expression Analysis

11.4.1 Sample Processing and DNA Extraction

For Aim 3, fecal samples from each child will be collected by the caregivers into sterile, screw-capped tubes containing RNA*Later* and frozen within 24 hours of collection in a home freezer. The participants will be instructed on how to collect the samples using protocols from the Fraser laboratory and be provided with written, illustrated instructions. The research staff will review these instructions with the caregivers during enrollment (Appendix, IND protocol). Immediately after collection and freezing, a member of the research team will be alerted to retrieve the samples and transport them to the laboratory. The stool will be stored at -40°C until transport to the University of Maryland. All research staff will receive certification on handling procedures.

Stool samples will be thawed on ice to remove representative 1 gram samples for DNA and RNA extraction as previously described.[109] These methods provide high quality nucleic acids for microbiome analysis on the Illumina sequencing platforms (see below).

11.4.2 Microbiome Community Profiling

16S rRNA gene amplification, sequencing and raw 16S rRNA data processing: The gut microbiota will be characterized by sequencing bacterial 16S rRNA gene amplicons from stool samples. A region of ~469 bp encompassing the 16S V3-V4 hypervariable regions[110-113] will

be targeted for sequencing using MiSeq's 300 bp paired-end sequencing reads and the two universal PCR primers 319F and 806R,[114] according to procedures previously published at The Institute for Genome Sciences (IGS) at the University of Maryland School of Medicine.[115, 116] Barcoding[114] will be used to multiplex >400 samples per MiSeq run, which will provide a sampling depth (~20,000 read pairs) sufficient to characterize the microbiota with high resolution. Raw 16S rRNA reads will be processed using published protocols.[114, 117, 118] Within- (alpha-diversity) and between- (beta-diversity) sample comparisons will then be performed using QIIME.[119] Enterotype classification will be carried out using the methods of Arumugan[120], as modified by the Fraser laboratory.

Microbiota will be compared cross-sectionally and longitudinally, using parameters such as diversity and evenness (Shannon, Simpson index), and similarity (phylogenetic UniFrac distance, Jensen-Shannon divergence), determined using standard tools, such as Mothur,[121] QIIME,[119] and statistical R package (www.r-project.org). In addition, statistically significant correlations between specific microbiota members and post-BB-12 time points, subject clusters, and clinical outcomes will be determined using bioinformatics tools, such as eLSA,[122] Metastats,[123] and LEfSe.[124] Since there is no universal standard for such correlations, we will use all existing statistical tools developed to analyze 16S rRNA gene datasets. We will incorporate in our analysis any new statistical tool developed during the course of the study by either IGS researchers or external laboratories. The 16S dataset will also be used to simulate microbiota functional compositions using PiCRUSt,[125] which uses available genome sequence data to infer functional gene contents based on the 16S rRNA microbiota compositions. This method will provide an alternative access to functional microbiota characterizations.

11.4.3 Anticipated Results and Interpretation

The experiments described in this aim will provide us with sequential samples from which data on both microbial composition (based of 16S rRNA profiling) and community function (based on RNASeq data) can be generated. We anticipate that longitudinal 16S rRNA profiling will provide insight into a number of distinct shifts in the microbiota (i.e., loss of particular taxa, loss of overall community diversity) that reflect the impact of antibiotics (control group) and antibiotics plus BB-12 (active yogurt group).

The impact of antibiotic treatment on the microbiota: In this clinical trial, all participants will be treated with a 7-10-day course of antibiotics beginning on day 1, and will be followed for another 20-23 days following cessation of antibiotic therapy. We will assess changes in the microbiota at five time points over this 30-day period. This study design will provide us an opportunity to capture information on antibiotic-induced changes in the microbiota, as well as monitor the recovery of the microbiota in 300 participants. We anticipate that a subset of participants 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 clinical outcome. To our knowledge, there have been no studies that examine the microbiota during the progression to a diarrheal state and the resolution of the diarrheal state. There have been limited studies on the treatment with antibiotics,[47, 126] but not in the context of disease resolution, nor in a pediatric population. We anticipate these findings will be novel and provide insight into the dynamics of the human gastrointestinal tract.

We will also use the baseline16S rRNA data to identify gut enterotypes in the 300 participants in this clinical trial. With regard to this aspect of data analysis, we will test the hypothesis that specific enterotypes are more/less resistant to development of antibiotic-induced diarrhea and more/less likely to show complete recovery of community structure following antibiotic

perturbation. To our knowledge, the impact of antibiotics on the gut microbiota has not been evaluated in the context of gut enterotypes and this will likely provide novel insights into differences in the stability of gut communities.

The impact of BB-12-supplemented yogurt on the microbiota: In addition to the observation of antibiotic-induced changes in the gut microbiota, we will also be able to examine the impact of concomitant administration of BB-12-supplemented yogurt over time in half of the participants. We will specifically look for differences between the control and active yogurt groups with regard to the degree of antibiotic-induced disturbance of the microbiota (i.e., loss of particular taxa, loss of overall community diversity) as well as the extent and time course of recovery of the microbiota following cessation of antibiotic therapy. It has been suggested that probiotics help to speed the recovery of the microbiota following antibiotic administration,[127] and this clinical trial will generate a significant amount of data to help to support or refute that assertion.

11.4.4 Metatranscriptomics

16S rRNA amplicon sequencing will provide an inventory of community members in the gut microbiota, whereas metatranscriptomics will provide an actual snapshot of the expressed genes -- moving the studies from descriptive to mechanistic. RNA will be isolated from the baseline and day 7 samples (funding limitations preclude metatranscriptome analysis of all samples; however, frozen samples will be available for future gene expression profiling studies if deemed to be of interest). We have budgeted for 100 samples to be analyzed, as we anticipate about 50 participants will have diarrhea, and the gene expression profiles of the microbiota at baseline and day 7 samples from these individuals will be analyzed.

Isolation of total RNA, host and bacterial rRNA depletion, mRNA amplification, library preparation and metatranscriptomic sequencing: Total RNA will be extracted from stools using an acid-phenol protocol, as described in Eloe-Fadrosh et al.[109] rRNA removal will be accomplished using the RiboZero Bacterial and Human rRNA Removal Kits, which consistently remove ~99% of all rRNA as estimated by the percent of RNAseq reads matching to any rRNA gene (SSU and LSU). The purified and rRNA-reduced RNA will be used to create indexed sequencing libraries to be multiplexed for sequencing. Analyses of the metatranscriptomic data will include mapping to reference genomes. We will use Bowtie to map sequence reads to our growing database of reference genome sequences that currently includes 1,482 and 1,941 non-redundant isolates.

Annotation and metabolic reconstructions: Reads that map to reference genomes will be converted into contigs on which the reference genome annotations will be transferred, and subsequently used for metabolic reconstruction using the Pathway Tools package developed by Dr. Peter Karp for the semi-automated prediction of metabolic pathways and full pathways visualization via web browser. In addition, HUMAnN2 (HMP Unified Metabolic Analysis Network)[128] will be used for accurate determination of presence/absence of microbial pathways, in the form of abundance tables summarizing gene families and metabolic pathways present in a given microbial community. This facilitates the analysis of a collection of metatranscriptomes as a matrix of gene/pathway abundances, similar to a collection of microarrays. Differential gene expression analysis will be carried out, as described in Eloe-Fadrosh et al.[109]

11.4.5 Anticipated Results and Interpretation

Gene expression and pathway reconstruction for the community: The metatranscriptomic studies will provide information about the gene expression profiles of all members of the microbiota as well as the *in vivo* expression profile for the probiotic, BB-12. Together, these data

will provide insight into (i) which organisms are metabolically active in the presence of antibiotic with and without BB-12; and (ii), whether the gene expression profile of BB-12 is similar in all patients. We anticipate that we will identify different transcriptional profiles that are associated with (i) administration of antibiotic alone or (ii) the concomitant administration of antibiotics and yogurt in subjects that either develop AAD or do not. What is not known is whether these transcriptional programs will differ depending on clinical outcome and/or the starting composition of the microbiota and/or the ingestion of BB-12. It is possible that each gut enterotype will exhibit a differential response to BB-12, in terms of changes in community composition, and/or gene expression. Such a finding will be of value in understanding the parameters that identify those subjects most likely to respond to probiotic intervention.

Antibiotic resistance gene expression profiles: With the increase in prevalence of antimicrobial resistance among bacterial pathogens and in environmental samples, investigators at IGS have developed bioinformatics pipelines for the identification of resistance genes in metagenomic/metatranscriptomic samples. The current iteration of the analysis pipeline identifies a total of 121 reference markers (representing 357 allelic variants) in the proposed reference database, based either on the screening of previously published literature or based on our own ongoing work in this field. Therefore, we have the ability to identify antibiotic-induced increases in resistance gene expression in the metatranscriptomics dataset and we will include this information as part of the analysis. Collectively, the work to be carried out as part of Aim 3 will provide the most robust description of metatranscriptome changes in association with concomitant antibiotic and probiotic administration to date.

11.4.6 Integration of Data

We will use MDI – a Bayesian method for unsupervised integration of multiple datasets – to look for correlations between clinical outcomes, 16S rRNA and gene expression data.[129] Each dataset will be modeled using a Dirichelet-multinomial allocation mixture model, with dependencies between models captured via parameters that describe agreement between the datasets. This approach has previously been used successfully to integrate multiple -omics datasets.

12 ETHICS/PROTECTION OF HUMAN SUBJECTS

12.1 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).

12.2 Institutional Review Board

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. Georgetown University's IRB operates under the appropriate Federal Regulations, and the PI, all co-investigators and research assistants have taken required classes on human subject research, ethics and Health Insurance Portability and Accountability Act (HIPAA).

This research will be conducted at Georgetown University and Pennsylvania State University. The initial production of the yogurt will be under the supervision of Pennsylvania State University. Pennsylvania State University has already granted permission to conduct sensory analysis tests of the yogurt.

The responsible official at the Georgetown University's IRB will sign 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.

12.3 Informed Consent Process

Informed consent is a process that is initiated prior to the parent/caregiver's agreeing to have their child participate in the study and continuing throughout the study participation. Extensive discussion of risks and possible benefits of this therapy will be provided to the parents/caregivers. Consent forms describing in detail the study interventions/products, study procedures, and risks are given to the parents/caregivers and written documentation of informed consent is required prior to starting intervention/administering study product.

After screening the participants, research personnel will meet with the parent/caregiver and administer the informed consent process. Signatures on hard copies of consent documents will be obtained.

When the enrollment/baseline visit is completed virtually, the informed consent process will be completed via voice call or Zoom video conference, either with e-signatures obtained through the Georgetown-licensed version of DocuSign or with hard copy signatures obtained. When hard copy signatures are obtained following a virtual enrollment/baseline visit, hard copies will be delivered to participant homes (in mailboxes and through mail slots, for example), such that participants can sign and return the documents without direct interaction with study staff.

To verify their identity and the identity of the participant before beginning the virtual consent process, parents/guardians will be asked to confirm their names, participants' names, and where they heard about the study/who referred them. Parents/guardians of prospective participants will be asked to confirm that they are comfortable discussing protected health

information over the phone and that they are using the phone line they prefer. Those with access to Zoom will be offered the option of completing the informed consent process via video conference, during which copies of consent documents (ICF, HIPAA, and assent where applicable) would be shared via the screensharing feature. Those without Zoom access or who prefer voice call will be emailed copies of consent documents.

The principal investigator will review, initial and sign each consent form after it is signed by the parent. If appropriate, an assent process may be done with older children (see Section 7.5 for details).

12.4 Inclusion of Females and Minorities

All children in reasonably good health except for their respiratory infection symptoms between the ages of 3 and 12 years who meet the inclusion/exclusion criteria, regardless of religion, sex, or ethnic background will be enrolled if their parents/caregivers sign the informed consent. Recruitment will also be done in Spanish and all forms translated.

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.

12.5 Subject Confidentiality

Subject confidentiality is strictly held in trust by the investigator, 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 participating subjects.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

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

13 DATA HANDLING AND RECORD KEEPING

The principal investigator is responsible to ensure the accuracy, completeness, legibility, and timeliness of the data reported. All source documents are to be completed in a neat, legible manner to ensure accurate interpretation of data. Changes or corrections will be made by crossing out the original entry with a single line, and initialing and dating the change.

13.1 Data Management Responsibilities

Data Coordination will be provided by the biostatistician and the CAPRICORN research network.

The following data collection forms will be double-entered into the database:
- Adverse Event Report Form (AE)
- Baseline Health Status (BH)
- Daily Assessment Diary (DD)
- Demographic Information Form (DI)
- Follow-up Form (FU)
- Inclusion/Exclusion Form (IE)
- Randomization Form (RZ)
- Serious Adverse Event Report Form (SA)

The following data collection forms will be single-entered into the database:
- Day 180 Follow-up Form (OF)
- Diarrhea Report Form (DR)
- Diarrhea Follow-up Form (DF)
- Diet Recall Diary (DT)
- Informed Consent Process Documentation (IP)
- Missed Contact Report Form (MC)
- PedsQL (Pediatric Quality of Life Inventory) (PQ)
- Sample Collection Form (SC)
- Withdrawal from Intervention Form (WI)
- Yogurt Consumption Follow Up (YC)

All of these forms will be used as source documents for the study. There are 18 data collection forms in total.

Each form has a two character abbreviation that will be used throughout the study to identify the forms. The data system uses this abbreviation to identify the form. The abbreviation, plus a revision number, must be entered into the system to identify every form prior to entry. The revision number refers to changes in forms that may occur after the study starts. At the start of the study, all forms have revision number 1, i.e. the Adverse Event form is referred to as AE1.

The databases will be created using REDCap [130]. All data and forms entered will be automatically validated upon entry into REDCap (range, type, and branching logic). Using the Double Data Entry Module, the data entry system is designed for independent double data entry—where the eight specified forms would be entered twice by two different RAs. This method of data entry helps to ensure data quality and accuracy. The data coordinator will provide two separate entry records and therefore two separate databases per Study ID:

Database 1 will be considered the primary database and Database 2 will be considered the secondary database. Each RA will be given access to only 1 entry record per Study ID. Each

month, the data coordinator will compare the values entered in the two databases and will provide the study site with a list of any existing differences. The Research Personnel will then be responsible for resolving the differences (for example, by checking the hard copy form) and making the necessary changes in the database. In case of an unresolved discrepancy between the two databases, Database 1 will be considered the final database.

The two data entry files are to be stored on a secure network location managed by Georgetown University Information Systems. Only members of the study team should have access to the project databases. The data system is designed to allow multiple users at a time, but the RA designated as Person #1 may only use Database 1 while the RA designated as Person #2 may only use Database 2. These 2 users will not be able to view or enter the same database at the same time.

The data entry system is designed to allow all information contained on a single form to be entered in a single keying session. It is always recommended to enter any one form in its entirety rather than breaking up a single form into multiple keying sessions. The data collection forms were also designed for keying directly into the data entry system without the need for additional transcription.

The data entry system has some built-in checks to ensure data accuracy as the RA is keying in data. These include range, format, arithmetic, and branching logic checks. However, there may be instances in which the RA finds that s/he repeatedly needs to enter values that are not allowed by the built-in checks of the data system. If this situation arises, the data coordinator should be contacted, and the data entry system may be changed if needed.

Always enter the data recorded on the forms, even if known to be wrong. Refer to the “special characters” section of the data entry system manual in the MOP for information on how to deal with suspect data. In addition, changes to records in the study database should never be made unless the changes have been made on the hard copy form. When making changes to data on the hard copy form, draw a single line through the old data, write in the new data, then initial and date the edit. The documentation should be sufficient to allow one to reconstruct the sequence of events leading to the change, starting with the original value.

All users will be assigned User IDs and passwords in order to gain entry to the data system. Every time the RA uses the “add a form” or data entry function, the system will automatically log and timestamp the entry. This is in order to track which forms were entered by which RA, and also in order to ensure that the independent double data entry is being conducted properly. RAs must be careful to enter their own Staff ID, *not* the Staff ID corresponding to the RA who filled out the form being entered.

13.2 Data Quality Control

Data quality control is a joint effort between the study RAs, PI, the biostatistician and CAPRICORN. Data quality control begins with clear data collection instructions. All study RAs will attend training to learn appropriate data collection procedures. These trainings will be lead by the PI and biostatistician with support from CAPRICORN to conduct training on data quality control.

Performance reports will be sent to the PI 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 PI can use these reports to gauge how they are performing in the study.

CAPRICORN will also conduct source verification audits at GU. At least once during the study, personnel from CAPRICORN will schedule a study visit. They will randomly select 3 active participants at the site and will do a complete audit to compare data in the study database to data on the source documents. Study personnel will not be notified of the 3 charts to pull until the day of the site visit, so they must be prepared to pull all source documents. The visit is expected to last one day and will be conducted by CAPRICORN personnel. During the visit, they will also check that all participants have signed informed consents, check any lab procedures and records, verify any measurement tool specifications, and ensure that the Research Personnel have up-to-date study documentation available. They will provide a written audit report to the PI within seven days of the site visit. This will document all findings from the audit. The PI will send a response letter to CAPRICORN within two weeks of receiving the audit report to document the response.

13.3 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.

13.4 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 subject, the investigator, 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 PI/study personnel is responsible for knowing and adhering to their IRB requirements.

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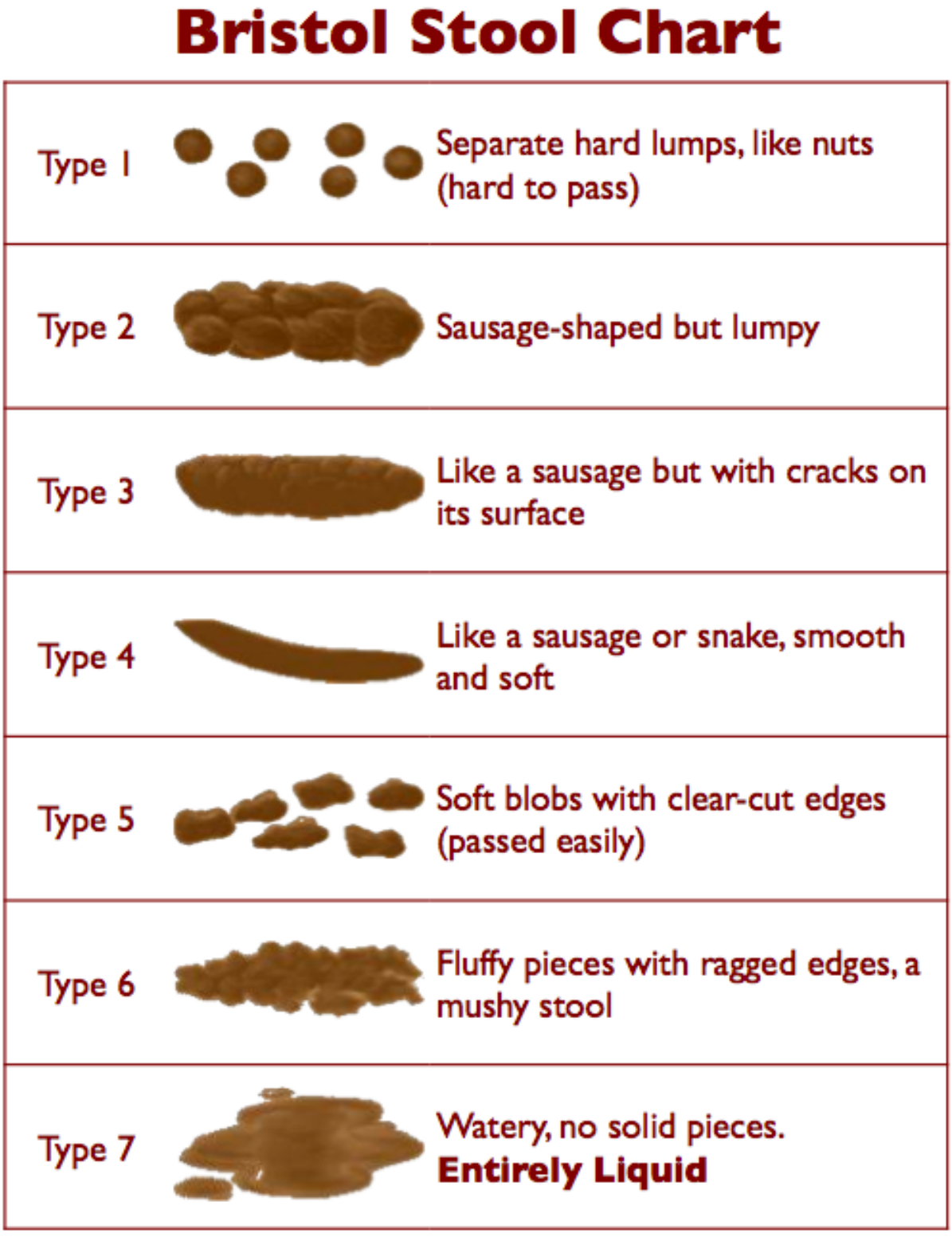
APPENDIX I.



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APPENDIX II. SUBSTUDY ADDENDUM

Project: The Impact of Socioeconomic Status on Clinical Trial Protocol Adherence. A Probiotic Randomized Controlled Study in Preventing Antibiotic Associated Diarrhea.

This project uses existing participant data in the PLAY ON study that participants have already agreed to release for research purposes. This will be a substudy of the PLAY ON study to allow us to better understand compliance. The aim of the project is to look at participant compliance with different aspects of the study protocol. Specifically, participant compliance is being studied in relation to socioeconomic status.

Participant's answers regarding how many doses of yogurt they consumed, date they completed their follow ups, date they collected samples, and presence of diet as well as daily diaries will be reviewed. In addition, the number of antibiotic doses completed will be examined. Using the data collected, a compliance scoring system will be generated. An average compliance score of participants in each category, indicating their level of compliance, will be calculated and looked at within differing socioeconomic brackets. We hope this information will allow us and other research groups suggestions on improving compliance.

Protocol Revision History

Version Number: 1.6
Version Date: July 28, 2020
Summary of Revisions Made: Addition of exclusion criterion, blood oxygen saturation <90% (if enrollment/baseline visit is completed in person and if the participant was prescribed antibiotics during a telemedicine visit) (page 27).

Version Number: 1.7
Version Date: July 28, 2021
Summary of Revisions Made: Addition of Substudy Addendum as Appendix II. Created new section, “Protocol Revision History.”