# Prebiotic GOS and lactoferrin for beneficial gut microbiota with iron supplements (PREFER)

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Study Protocol

Version 4

21 February 2020

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## TRIAL REGISTRATION DATA

**Primary Registry and Trial Identifying Number:** ClinicalTrials.gov: NCT03866837.

**Date of Registration in Primary Registry**: March 7, 2019

**Secondary Identifying Numbers**: National Institute of Diabetes and Digestive and Kidney Diseases Grant Number R01 DK115449
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**Source of Financial Support**: National Institute of Diabetes and Digestive and Kidney Diseases

**Protocol Title**: Prebiotic GOS and lactoferrin for beneficial gut microbiota with iron supplements

**Country of Recruitment**: Kenya

**Health Problem Studied**: Infant dysbiosis with iron supplementation

**Intervention**: Each study group will receive in-home fortification for 6 months with multiple micronutrient powders with 5 mg iron (as ferrous fumarate [5.0 mg]) and

**Study group A**: Galacto-oligosaccharides, 7.5 mg.
**Study group B**: Bovine lactoferrin, 1.0 g.
**Study group C**: Galacto-oligosaccharides, 7.5 mg, and bovine lactoferrin, 1.0 g.
**Study group D**: No galacto-oligosaccharides or bovine lactoferrin (Placebo)

The multiple micronutrient powders are composed of Vitamin A, 400 **µ**g; Vitamin D, 5 **µ**g; Vitamin E, 5 mg; Thiamine, 0.5 mg; Riboflavin, 0.5 mg; Vitamin B6, 0.5 mg; Folic Acid, 90 **µ**g; Niacin, 6 mg; Vitamin B12, 0.9 **µ**g; Vitamin C, 30 mg; Copper, 0.56 mg; Iodine, 90 **µ**g; Selenium, 17 **µ**g; Zinc, 4.1 mg; Phytase, 190 FTU; Iron, 5 mg (as ferrous fumarate [5.0 mg]). In the intervention for Study Group D, the usual maltodextrin carrier will be added to a weight of 10.0 g. In Study Groups A and C, galacto-oligosaccharides will replace the maltodextrin and will be added as 10.0 g Vivinal GOS 75 Powder (Friesland Campina, Wageningen, The Netherlands) containing 7.5 g of galactooligosaccharides. This dose of 7.5 g/day was selected based on studies that reported a bifidogenic effect in infants and adults at doses from 2.5 to 10 g/day97 and the typical daily dose delivered by commercial infant formula containing galacto-oligosaccharides. In Study Groups B and C bovine lactoferrin (Friesland Campina, >90% apo-lactoferrin), 1.0 g will be added. The dose of lactoferrin was chosen based on the estimated amount consumed by a breastfeeding 12-month-old infant.88 The micronutrient powders will be packed in identical appearing group-coded sachets (each containing one daily dose) at the factory, and the codes will be kept solely by the Data and Safety Monitoring Board (DSMB) and the producer; study participants and investigators will be blinded to group assignment.

**Inclusion and Exclusion Criteria**: The study will be conducted at the Msambweni District Hospital, Msambweni, Kenya. The population to be studied will consist of infants, 6 months (± 3 weeks) of age with both infant and mother in good health, and free of any acute or chronic illness.

Inclusion criteria will be:

(i) delivery vaginally or by cesarean section,
(ii) an infant age of 6 months (±3 weeks),
(iii) mother ≥15 years of age,
(iv) infant still breastfeeding, and
(v) anticipated residence in the area for the study duration.

Exclusion criteria will be:

(i) inability to provide informed consent,
(ii) hemoglobin < 70 g/L,
(iii) Z scores for weight-for-age (WAZ) or weight-for-height (WHZ) <3,
(iv) any maternal or infant chronic illness,
(v) administration of any vitamin or mineral supplements for the past 2 months, and
(vi) any history of antibiotic treatment in the past seven days.

**Study Characteristics**: This will be a single-center, randomized, controlled double-blind 9-month trial with a 2X2 factorial design to determine the efficacy of galacto-oligosaccharides and bovine lactoferrin in preserving a beneficial gut microbiota during iron supplementation in Kenyan infants. In a coordinated fashion, we will examine microbiota composition, metabolic activity, and inflammatory potential *in vitro* with treatments paralleling those in the clinical trial, using immobilized fecal microbiota from Kenyan infants to inoculate our long-term continuous polyfermenter intestinal model *in vitro* in conjunction with cellular studies.

The research plan has been designed to coordinate the conduct of the randomized clinical trial with studies in intestinal fermentation and cellular models. In the infant studies, the randomized, controlled double-blind 9-month clinical trial will enroll 6-month old Kenyan infants into groups receiving in-home fortification with multiple micronutrient powders with iron and (i) GOS, (ii) bLF, (iii) GOS and bLF, and (iv) no GOS or bLF for 6 months; each infant will then be followed for an additional 3 months to determine the longer term effects of the treatments. The study design does not include a group given micronutrient powders without iron because our previous studies have shown that no decrease in anemia or improvement in hemoglobin concentration is found in groups of infants given micronutrient powders without iron. Based on our series of previous clinical trials in southern coastal Kenya, screening of infants followed by enrollment of 2 per week, or 8 infants each month, is a realistic rate for subject accrual. As detailed below, each enrolled infant is then visited weekly to deliver micronutrient powders and assess morbidity and protocol adherence with a questionnaire. At the end of the first 9-month period, 72 infants will have been enrolled constituting Study Cohort 1. As these infants complete their participation in the trial, enrollment of infants in Study Cohort 2 will begin, maintaining the number of weekly study visits at 72 infants per week to remain within the capacity of the Kenyan study team. To reach the needed sample size of 288 infants, enrollment of 4 study cohorts is planned, with randomized assignment to the 4 study groups within each study cohort. Enrollment of Study Cohort 1 will begin in Year 1, Month 6; of Study Cohort 2, in Year 2, Month 3; of Study Cohort 3, in Year 3, Month 1, and of Study Cohort 4, in Year 3, Month 9. The last subject will have completed all study procedures in Year 5, Month 3.

Stratified randomization will be carried out by the Trial Statistician using computer-generated randomly permuted blocks of size 2, 4 or 6 with stratification by gender and assignment of eligible

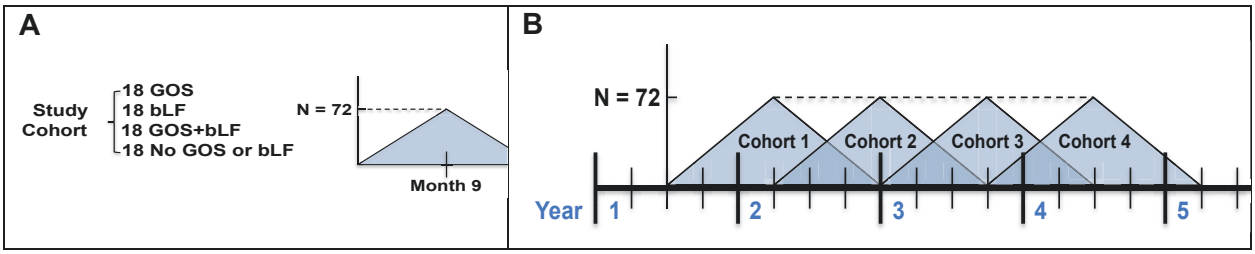
infants to one of the 4 intervention groups using 4 color codes. Allocation will be known solely by the Trial Statistician and the Clinical Trial Safety Officer and concealed from all other study personnel.

**Sample size**: Based on the preliminary data from our study with galacto-oligosaccharides (GOS), a sample size of 288 subjects (72 in each group) would permit detection of a minimum standard deviation of size 0.2 among the standardized means of the 4 groups with 80% power at the 0.05 significance level. In the preliminary data from the GOS study, the standard deviation of the ratios of harmful pathogens and beneficial bacteria in the study group receiving iron alone and the group receiving iron + GOS ranged from 0.002 to 0.1, so our study, with a sample size of 288 subjects, has 80% power to detect a standard deviation of 0.0004 to 0.02.

**Primary outcome**: The primary outcome measure will be the ratio of the abundances of potentially harmful (enteropathogenic and/or enterotoxigenic *E. coli, C. difficile*, members of the *C. perfringens group, B. cereus, S. aureus*, sum of *Shigella* spp., and *Salmonella*) to beneficial (bifidobacteria and the group of La*ctobacillus/Leuconostoc/Pediococcus* spp.) bacterial genera at 1 month.

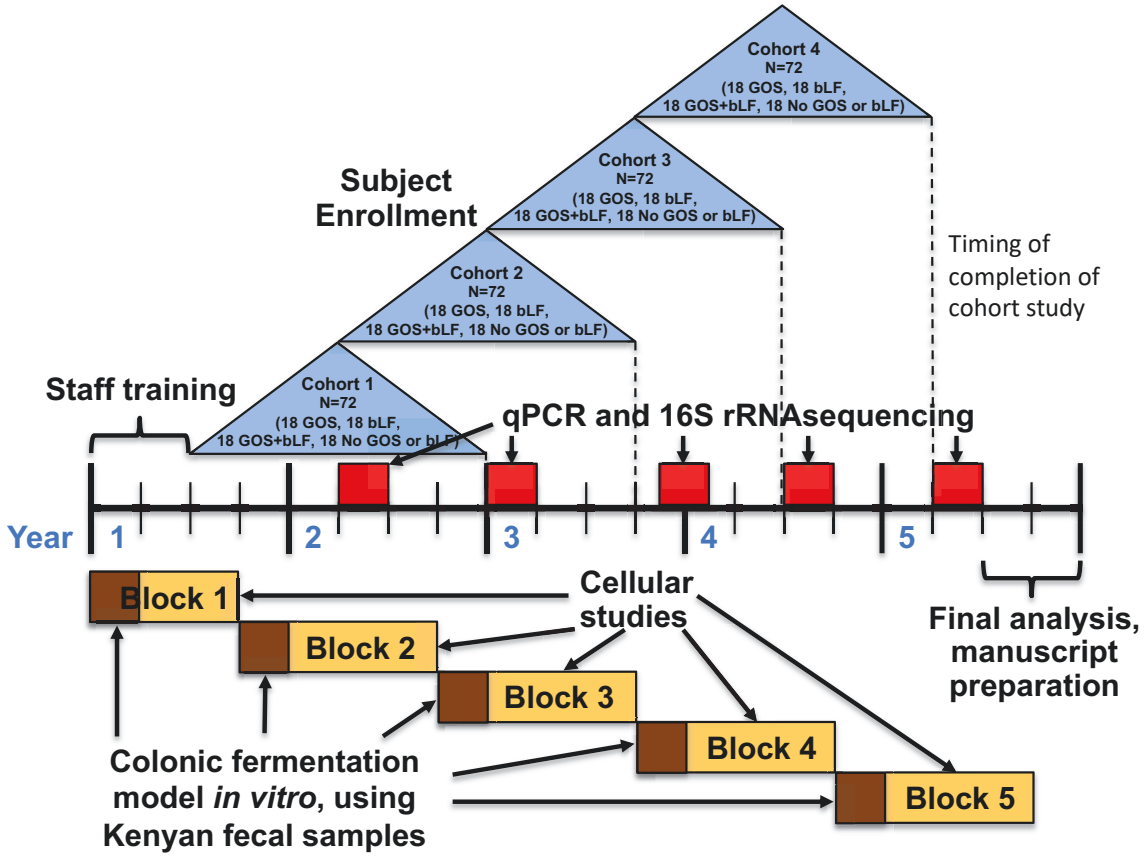
**Principal secondary outcomes**: The principal secondary outcome measures will include the ratio of the abundances of potentially harmful to beneficial bacterial genera ratios at 6 and 9 months and comparisons of microbiota composition at all time points, including assessment of effects of helminth infections. Other principal secondary outcomes will include the prevalence at 1, 6 and 9 months of diarrhea, malaria, anemia, iron deficiency, iron deficiency anemia, inflammation, respiratory tract infections, and other illnesses.

**Figure 1:** **A**. Randomization and rate of infant enrollment in each study cohort.
**B**. Schedule of enrollment of the 4 study cohorts.




The randomized, controlled double-blind 9-month clinical trial will enroll 6-month old Kenyan infants into groups receiving in-home fortification with multiple micronutrient powders with iron and (i) GOS, (ii) bLF, (iii) GOS and bLF, and (iv) no GOS or bLF for 6 months; each infant will then be followed for an additional 3 months to determine the longer-term effects of the treatments.

**Figure 2**: Coordination of the clinical trial with the colonic fermentation studies *in vitro*.



**Rationale and Background**:

The ultimate goal of this research is to develop a means to safely administer iron supplements to infants in settings with a high infection burden. The scientific premise underlying this project is that promoting development of a beneficial, protective gut microbiota by co-administration of prebiotic galacto-oligosaccharides (GOS) and iron-sequestering bovine lactoferrin (bLF) during iron supplementation will prevent iron-induced increases of opportunistic enteropathogens that cause infection and inflammation. We will conduct a randomized clinical trial in 6 month-old Kenyan infants in conjunction with mechanistic microbiota studies using our established long-term continuous polyfermenter platform inoculated with immobilized fecal microbiota from Kenyan infants. The period from about 6 months to 1 year of age is vital both for iron nutrition and for the establishment of a healthy gut microbiome that promotes immune system development, local immune homeostasis and limits pathogen colonization.

Iron deficiency, the principal cause of anemia globally, affects more than two billion individuals, predominantly infants, children and women of childbearing age. Iron deficiency impairs cognitive and behavioral development in childhood, compromises immune responsiveness, decreases physical performance, and when severe, increases mortality among infants, children and pregnant women. Effective prevention and treatment of iron deficiency uses iron supplements or fortificants to increase oral iron intake. Generally, only a small fraction of the added iron is absorbed in the upper small intestine, with 80% or more passing into the colon. Because iron is an essential micronutrient for growth, proliferation, and persistence for most intestinal microbes, the increase in iron availability has profound effects on the composition and metabolism of the intestinal microbiota. In particular, iron is a prime determinant of colonization and virulence for most enteric gram-negative bacteria, including *Salmonella*, *Shigella* and pathogenic *Escherichia coli*. Commensal intestinal microorganisms, principally of the genera *Bifidobacterium* and *Lactobacillus,* require little or no iron, provide a barrier effect and can inhibit pathogen growth by a variety of methods, including sequestration of iron, competition for nutrients and for intestinal epithelial sites, stabilization of intestinal barrier function, and production of antibacterial peptides and organic acids that lower the pH. Increases in unabsorbed iron can promote the growth of virulent enteropathogens that overwhelm barrier strains and disrupt the gut microbiota.

We were the first to document the iron-induced emergence of a potentially pathogenic intestinal microbiota (qPCR) in a six-month, randomized, double-blind, controlled trial in school-age children in the Côte d'Ivoire (Am J Clin Nutr. 2010; 92:1406-1415). In these anemic African children, iron supplementation was associated with a decrease in lactobacilli and significant increases in enterobacteria and in fecal calprotectin, a marker of gut inflammation. Subsequent studies with iron-containing micronutrient powders in Kenyan infants (qPCR, 16S rRNA sequencing) have found increases in pathogenic enterobacteria, decreases in bifidobacteria, and evidence of increases in intestinal inflammation. Our coordinate investigations *in vitro*, using fermentation and cellular models of the effects of iron deficiency and iron supplementation on the gut microbiota of infants and children, have shown large iron-related shifts toward a more pathogenic microbiota profile (Curr Opin Biotechnol 2015; 32: 149-155). After pneumonia, diarrhea is the leading cause of death in young African children. Oral iron supplements are associated with a significant 15% increase in the rate of diarrhea in children in malaria-endemic areas, as documented in the current Cochrane review (8 trials, 23,912 child-months). Our most recent study has shown that prebiotic galacto-oligosaccharides (GOS) can provide partial amelioration of the adverse effects of iron-induced dysbiosis by enhancing the growth of barrier populations of bifidobacteria and lactobacilli (Gut. 2015; 64:731-742). We hypothesize that the combination of prebiotic GOS with bovine lactoferrin (bLF), adding iron sequestration, antimicrobial and immunomodulatory activities, will provide virtually complete protection against the adverse effects of added iron on the intestinal microbiota. Our research has two specific aims:

(1) to conduct a randomized, controlled double-blind 9-month clinical trial in 6-month old Kenyan infants comparing the effects on gut microbiome composition among groups receiving in-home fortification for 6 months with micronutrient powders containing 5 mg iron (as ferrous fumarate [5.0 mg]]) and (i) galacto-oligosaccharides (GOS; 7.5 g), (ii) bovine lactoferrin (bLF, 1.0 g), (iii) GOS (7.5 g) and bLF (1.0 g), and (iv) no GOS or bLF. Each infant will then be followed for an additional 3 months to determine the longer-term effects of the treatments.

(2) to examine mechanisms of iron, prebiotic GOS and iron-sequestering bLF on microbiota composition, enteropathogen development, microbiota functions and metabolic activity, and inflammatory potential *in vitro* with treatments paralleling those in Specific Aim 1, using immobilized fecal microbiota from Kenyan infants to inoculate our established long-term continuous polyfermenter intestinal model (PolyFermS) to mimic Kenyan infant colon conditions, together with cellular studies.

Combining *in vivo* clinical and *in vitro* approaches will help guide formulation of safer iron supplements and fortificants and improve our understanding of the mechanisms whereby prebiotic GOS and iron-sequestering bLF support commensal microbiota to prevent iron-induced overgrowth by opportunistic enteropathogens.

This research is intended to use our improved insight into the development and functions of the gut microbiota to develop an innovative strategy to help solve a global public health problem, the safe treatment and prevention of iron deficiency in infants living in areas with a high infection burden. Reduction of iron-deficiency anemia in infants and young children is a chief priority of the World Health Organization (WHO). Iron deficiency, the most common micronutrient deficiency, is the dominant cause (≥60%) of anemia worldwide, impairs cognitive and behavioral development in childhood, perhaps irreversibly, compromises immune responsiveness, and increases mortality among infants and children. By 4 to 6 months of age, iron stores present at birth become depleted and, with the low concentration of iron in human milk (about 0.5 mg Fe/L, highly bioavailable), full-term, breastfed infants become increasingly dependent on complementary foods as a source of iron. The risk of iron deficiency then increases as iron needs for growth outpace the sparse amounts of bioavailable iron in complementary foods in most developing countries. In these areas, the WHO strongly recommends home fortification of foods with micronutrient powders containing iron to improve iron status and reduce anemia among infants but concerns about the safety of this approach are increasing. Less than 20% of the iron in micronutrient powders is absorbed, with the remainder passing into the colon. With a WHO-recommended supplemental amount of 12.5 mg Fe/day, roughly 10 mg of iron will enter the colon, a more than 25-fold increase over the 0.25 to 0.35 mg Fe/day supplied by breast milk.

In addition, functional maturation of the microbiome follows the introduction of complementary foods to infants at 4 to 6 months of age, with increased alpha-diversity (within an individual), reduced beta-diversity (among individuals), and a shift from the relative abundance of genes involved in the degradation of sugars from breast milk towards enrichment of genes involved in the degradation of complex sugars and starch. This period is critical for development of the reciprocal interactions between the infant host and the gut microbiota: the gut microbiota has evolutionarily conserved roles in the metabolism, immunity, development, and behavior of the host while the infant immune system is involved in shaping the composition of the commensal microbiota. While this evidence indicates that iron-induced dysbiosis has enduring effects on the gut microbiota, the primary goal of this project is the prevention of diarrhea and other infectious complications during iron supplementation in infancy. Iron, an essential micronutrient for both the infant and microbes, is the focus of competition between (i) the iron sequestration and scavenging mechanisms that underlie nutritional immunity in the infant and (ii) the siderophores and other

sophisticated iron acquisition strategies of pathogenic bacteria. Supplemental iron impairs the colonization resistance of the commensal microbiota while enhancing the proliferation and virulence of opportunistic enteropathogens. The overall significance of our research lies in the development of a strategy to counteract the adverse effects of supplemental iron in infants by combining a prebiotic that helps support the commensal microbiota (galacto-oligosaccharides [GOS]) with a milk protein, bovine lactoferrin (bLF), that can sequester iron, help prevent colonization and infection of the epithelium in the small intestine, and modulate both innate and adaptive immune responses.

Our research is based on the hypothesis that promoting development of a beneficial gut microbiota by co-administration of prebiotic GOS and iron-sequestering bLF can offset iron-supplement-induced increases in opportunistic enteropathogens. The diarrheagenic effects of iron supplementation in children in malaria-endemic areas are documented in the most recent Cochrane review: a 15% increase in diarrhea (eight trials, 23,912 child-months). In an area with a high infection burden in the absence of malaria (Pakistan), a randomized clinical trial in 2,746 children, 6 to 18 months of age, found that groups receiving a micronutrient powder with iron had an increased risk of diarrhea (P=0.001) and of bloody diarrhea (P=0.003).

We were the first to describe the emergence of a potentially pathogenic intestinal microbiota with iron supplementation (Am J Clin Nutr. 2010; 92:1406-1415). In a six-month, randomized, double-blind, controlled trial of iron supplementation in school-age children in the Côte d'Ivoire, we found that anemic children carried an unfavorable ratio of fecal enterobacteria to bifidobacteria and lactobacilli. Supplemental iron increased the abundance of enterobacteria (P<0.005), decreased lactobacilli (P<0.0001) and increased gut inflammation as evaluated by fecal calprotectin (P<0.01). We subsequently conducted the first randomized, double-blind, controlled trials of iron supplementation with micronutrient powders in African infants under conditions virtually identical to those now proposed at our research site in Kenya (Gut. 2015; 64:731-742). In brief, two trials were carried out in 6-month-old Kenyan infants (n=115) consuming maize porridge fortified with micronutrient powders daily for 4 months. In both trials, the abundances of enterobacteria were increased, especially of pathogenic *E. coli* strains (P<0.03). Both the enterobacteria/bifidobacteria ratio (P=0.02) and the fecal calprotectin concentration (P=0.003) were increased.

We also have published (Gut 2017;66:1956-1967) the results of a similarly designed 4-month, randomized controlled trial in Kenyan infants using a micronutrient powder combining GOS (7.5 g) with a low dose of iron (5 mg: 2.5 mg as NaFeEDTA and 2.5 mg as ferrous fumarate, with ascorbic acid and a phytase to enhance iron bioavailability). The 155 infants, aged 7 to 9 months at enrollment, were randomized to receive either the micronutrient powder with iron, iron and GOS, and no iron or GOS (control). As in our earlier trial in Kenya, no significant change in hemoglobin concentration was found after four months in the control group receiving the micronutrient powder without iron. In contrast, in the two groups receiving iron, the hemoglobin concentration increased by a mean of 6.9 g/L and the proportion of infants with anemia decreased by about 20% (P<0.001 for both). These results are comparable to those reported using the WHO-recommended 12.5 mg iron without ascorbic acid and phytase and are evidence that a micronutrient powder containing highly bioavailable low-dose iron together with prebiotic GOS is effective in reducing anemia. At baseline, 16S rRNA sequencing found a high prevalence of gut enteropathogens, including pathogenic *E. coli* in 77%, *Clostridium perfringens* in 65%, and *Clostridium difficile* in 35%. At 4 months, the group receiving the micronutrient powder with iron alone had increased mean abundances of enterobacteria with decreased bifidobacteria and lactobacilli, compared to the control group (P<0.05 for all). In contrast, compared to control, the group receiving iron with GOS had no significant differences in these taxa. We measured plasma intestinal-type fatty acid-binding protein (I-FABP) as an indicator of enterocyte injury. Compared

to the control group, I-FABP increased in both the group receiving iron alone (P=0.049) and the group receiving iron and GOS (P=0.058). Compared to control, our measure of gut inflammation, fecal calprotectin, increased in both the group receiving iron alone and the group receiving iron and GOS (P<0.05 for both). Together, these data provide evidence that a micronutrient powder with highly bioavailable low-dose iron together with prebiotic GOS mitigates the decrease in the beneficial barrier commensals caused by iron by maintaining levels of bifidobacteria and lactobacilli. Nonetheless, the protection against iron-induced adverse effects by GOS is incomplete. The combination of iron with GOS reduced abundances of enteropathogens compared to iron alone but most infants continued to carry high abundances of enteropathogens at 3 weeks and 4 months. Compared to the control group, fecal calprotectin and plasma I-FABP remained elevated at 4 months in both the iron alone and the iron and GOS groups, evidence of ongoing inflammation and enterocyte damage associated with iron in the micronutrient powder, despite added GOS.

We have established a novel continuous intestinal fermentation model *in vitro* and, together with cellular and animal studies, used these tools to examine the complex effects of iron on gut microbiota, intestinal health and infections (Curr Opin Biotechnol. 2015; 32:149-155). Our procedure is to carefully compare model results *in vitro* with clinical data *in vivo* with respect to microbial composition, functions and dynamic responses to perturbations. Special care and attention are devoted to the selection of the fecal donor, application of protective conditions, anaerobiosis, and handling of fecal material from donor to reactor, as well as to the techniques used for inoculation, reactivation and cultivation of the gut microbiota. Our models, which we have termed the PolyFermS platform, aim to reproduce both the planktonic (free-cell) and sessile (biofilm-associated) bacterial populations, with bacterial composition and density mimicking that of the colon. These models are composed of a first-stage inoculum reactor seeded with immobilized fecal microbiota and used to constantly inoculate parallel-operated systems that are designed to mimic different sections of the colon. These fermentation models can be arranged in a variety of configurations, allowing the comparison of different treatment effects with an untreated control inoculated with the same microbiota. Our novel PolyFermS platform is currently recognized as one of the most accurate gut fermentation model for *in vitro* mechanistic studies.

Lactoferrin (LF): Lactoferrin, the second most abundant protein in human milk whey, is a multifunctional iron-binding protein with concentrations ranging from 6 g/L in early milk (<28 days lactation) to 1 to 2 g/L in mature milk, with more than 90% in the apo- (iron-free) form. Resistant to digestion, intact lactoferrin is found in substantial amounts in the feces of breastfed infants. Lactoferrin-bound iron is absorbed through an intestinal lactoferrin receptor. Bovine lactoferrin (bLF) is highly homologous with human lactoferrin. Lactoferrin has a molecular weight of about 80,000 with a single polypeptide chain (~690 amino acid residues) folded into two globular lobes, each of which can bind a single iron atom with high affinity (a binding constant of ~$10^{20}$, similar to that of transferrin). While transferrin releases iron at a pH of ~5.5, lactoferrin retains iron to a pH of ~3.5 and can bind iron at the pH values found throughout the small and large intestine, making lactoferrin an effective antioxidant and a bacteriostatic agent for withholding iron from microbes. Lactoferrin has a variety of other functions, including growth-promoting effects on bifidobacteria, bactericidal and anti-inflammatory activities, and modulation of immune function through a several mechanisms, including binding to specific sites on DNA, and acting as a transcription factor for a variety of cytokines.

Clinical studies provide evidence for the efficacy of iron-sequestering lactoferrin. A recent Cochrane review concluded that oral bLF decreases late-onset sepsis and necrotizing enterocolitis in preterm infants without adverse effects (Cochrane Database Syst Rev 2015; CD007137). A randomized, double-blind controlled trial of bLF, 0.5 g twice daily, for prevention of diarrhea in 555 children, 12 to 18 months of age, found reductions in the longitudinal prevalence

(P=0.017), duration (P=0.046) and severity of episodes (J Pediatr 2013; 162: 349-356). Most importantly, a recent randomized, double-blind, controlled clinical trial evaluated the effect of bLF with iron supplementation. bLF (380 mg/1000 g milk) and placebo were given to 260 infants 4 to 6 months of age, who were weaned to feeding with iron-fortified formula (40 mg Fe/1000 g milk) (Nutrition 2016; 32: 222-227). For a formula volume of about 750 mL/day, the formula provided an additional 30 mg Fe/day. bLF reduced both the incidence (rate ratio 0.32, 95% CI 0.20-0.72) and duration (rate ratio 0.39, 95% CI 0.22-0.78) of diarrhea (P<0.05 for both). This result provides evidence that iron-sequestering bLF alone is effective in reducing the incidence and duration of diarrhea in infants given supplemental iron.

## Study methods:

**Overall strategy, methodology, and analyses:** The ultimate goal of this research is to develop a means to safely administer iron supplements to infants in settings with a high infection burden. Our approach is to enable home fortification of foods with micronutrient powders containing iron to improve iron status and reduce anemia among infants, as is strongly recommended by World Health Organization (WHO) guidelines. Aware that oral iron supplements are associated with a significant 15% increase in the risk of diarrhea in children in malaria-endemic areas, as documented in the most recent Cochrane review (Cochrane Database Syst Rev 2016; 2: CD006589), our overall strategy is to promote development of a beneficial, protective gut microbiota by co-administration of prebiotic galacto-oligosaccharides (GOS) and iron-sequestering bovine lactoferrin (bLF) during iron supplementation to prevent iron-induced increases of opportunistic enteropathogens causing infection and inflammation. As shown in our recently published study (Gut. 2015; 64:731-742), prebiotic GOS selectively enhances the abundances of beneficial bifidobacteria and lactobacilli**.** The scientific premise of this project is that combining prebiotic GOS with iron-sequestering bLF, adding iron-withholding, antibacterial, and immunomodulatory activities, can provide almost complete protection against the adverse effects of added iron on the intestinal microbiota. We will conduct a randomized clinical trial in Kenyan infants in conjunction with mechanistic microbiota studies using our established PolyFermS platform inoculated with immobilized fecal microbiota from Kenyan infants. Our primary analyses will focus on the effects of GOS, bLF, and their combination on the composition of the infant gut microbiome, using a two-part mixed-effects regression model for analyzing longitudinal microbiome compositional data.

**Overview of research plan and coordination of studies:** The research plan has been designed to coordinate the conduct of the randomized clinical trial (Specific Aim 1) with the studies in intestinal fermentation and cellular models (Specific Aim 2). In Specific Aim 1, the randomized, controlled double-blind 9-month clinical trial will enroll 6-month old Kenyan infants into groups receiving in-home fortification with multiple micronutrient powders with iron and (i) GOS, (ii) bLF, (iii) GOS and bLF, and (iv) no GOS or bLF for 6 months; each infant will then be followed for an additional 3 months to determine the longer-term effects of the treatments. **The study design does not include a group given micronutrient powders without iron because our previous studies have shown that no decrease in anemia or improvement in hemoglobin concentration is found in groups of infants given micronutrient powders without iron.** Based on our series of previous clinical trials in southern coastal Kenya over the past 6 years, screening of infants followed by enrollment of 2 per week, or 8 infants each month, is a realistic rate for subject accrual. As detailed below, each enrolled infant is then visited weekly to deliver micronutrient powders and assess morbidity and protocol adherence with a questionnaire. At the end of the first 9-month period, 72 infants will have been enrolled (Figure 1A), constituting Study Cohort 1. As these infants complete their participation in the trial, enrollment of infants in Study Cohort 2 will begin, maintaining the number of weekly study visits at 72 infants per week to remain within the capacity of the Kenyan study team. To reach the needed sample size of 288 infants, enrollment of 4 study cohorts is planned, with randomized assignment to the 4 study groups *within* each study cohort. Figure 1B shows the schedule of enrollment of the 4 study cohorts; enrollment of Study Cohort 1 will begin in Year 1, Month 6; of Study Cohort 2, in Year 2, Month 3; of Study Cohort 3, in Year 3, Month 1, and of Study Cohort 4, in Year 3, Month 9. The last subject will have completed all study procedures in Year 5, Month 3.

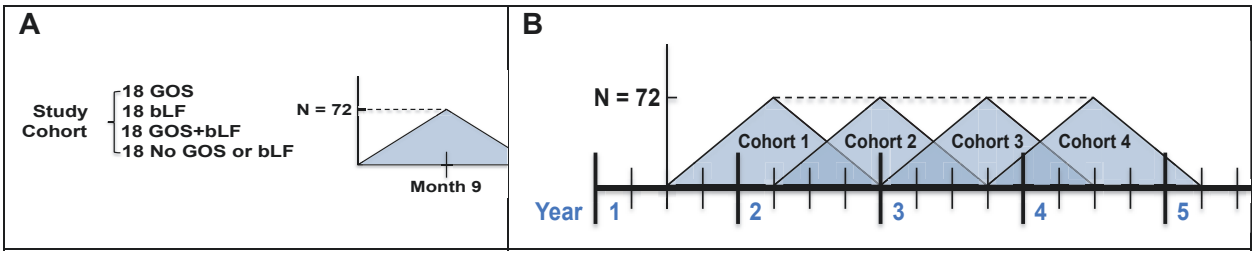



**Figure 1**: **A**. Randomization and rate of infant enrollment in each study cohort.
**B**. Schedule of enrollment of the 4 study cohorts.

The coordination of the clinical trial studies in Specific Aim 1 with the colonic fermentation studies in Specific Aim 2 is shown in Figure 2. For Specific Aim 1, the first six months of Year 1 will be devoted to the development of the Manual of Operations, sensory testing, and training of the staff in the Msambweni District Hospital in study procedures. Enrollment of the study cohorts will then begin. For each infant, stool and venous blood samples will be collected at enrollment and at 1, 6 and 9 months.

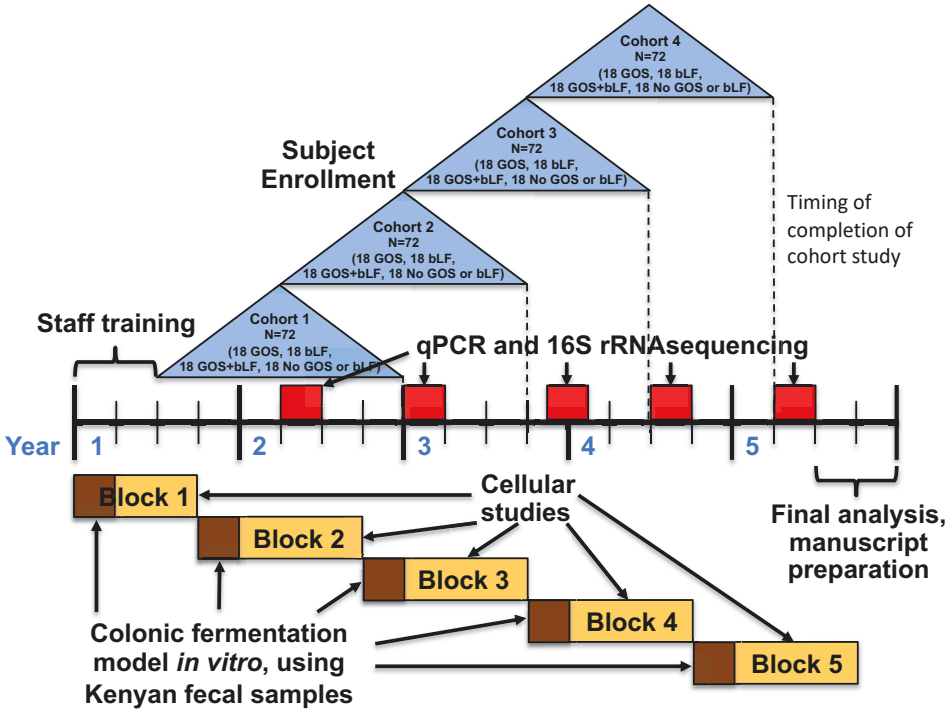



**Figure 2**: Coordination of the studies in Specific Aims 1 and 2.

After each Study Cohort completes collection of the enrollment and 1 month stool sample, and subsequently, after each subject completes collection of the 6- and 9-month stool samples, targeted qPCR, and measurements of fecal pH, short-chain fatty acids, calprotectin, plasma intestinal fatty acid binding protein (I-FABP) and preparation and storage of DNA will be carried out, along with interim 16S rRNA sequencing. After all stool samples have been collected and prepared, a final 16S rRNA sequencing will be completed in a single analysis in Year 5, Months 3 to 6.

For Specific Aim 2, stool samples from representative study subjects prior to randomization will be collected anaerobically with protective precautions to preserve the composition and

functionality of the gut microbes (see above), flown directly to the Laboratory of Food Biotechnology, ETH-Zürich, and immediately used to prepare fecal beads for inoculation of our continuous colonic fermentation model. For Block 1, 8 fecal samples, and for Blocks 2 through 5, 4 fecal samples will be studied in each Block.

Specific Aim 1: to conduct a randomized, controlled double-blind 9-month clinical trial in 6-month old Kenyan infants comparing the effects on gut microbiome composition among groups receiving in-home fortification for 6 months with micronutrient powders containing 5 mg iron (as ferrous fumarate [5.0 mg]) and (i) galacto-oligosaccharides (GOS; 7.5 g), (ii) bovine lactoferrin (bLF; 1.0 g), (iii) GOS (7.5 g) and bLF (1.0 g), and (iv) no GOS or bLF. Each infant will then be followed for an additional 3 months to determine the longer-term effects of the treatments.

**Study hypotheses:** Our primary study hypothesis is that co-administration of prebiotic GOS and iron-sequestering bLF in multiple micronutrient powders with iron that are used for in-home fortification will promote development of a beneficial, protective gut microbiota and prevent iron-induced increases of opportunistic enteropathogens. Key secondary hypotheses are that the iron-sequestering, antibacterial, and immunomodulatory activities of bLF will be *synergistic* with GOS (i) in promoting a beneficial gut microbiota, and (ii) in decreasing the incidence and duration of diarrhea.

**Overview and study registration:** This will be a single-center, randomized, controlled double-blind 9-month trial with a 2X2 factorial design to determine the efficacy of prebiotic GOS and iron-sequestering bLF in preserving a beneficial gut microbiota during iron supplementation in Kenyan infants. Study registration will be at ClinicalTrials.gov.

**Study setting**: The clinical trial will be conducted in the catchment area of the Kikoneni health center in southern coastal Kenya, a malaria-endemic region[1] that is the site of our studies over the past 6 years of micronutrient powders with iron and of our recently published studies with GOS. The typical local weaning food is a liquid maize porridge, 'uji'; a regular portion consists of about 8 to 10 g of maize flour boiled in 100–150 mL water and sweetened with sugar. Kenya has a national school-based deworming program for soil-transmitted helminths but the risk of infection remains considerable, even among infants 6 to 15 months of age.

**Inclusion and Exclusion Criteria**: The study will be conducted at the Msambweni District Hospital, Msambweni, Kenya. The population to be studied will consist of infants, 6 months (± 3 weeks) of age with both infant and mother in good health, and free of any acute or chronic illness.

Inclusion criteria will be:

(i) vaginal or cesarean delivery,
(ii) an infant age of 6 months (±3 weeks),
(iii) mother ≥15 years of age,
(iv) infant still breastfeeding, and
(v) anticipated residence in the area for the study duration.

Exclusion criteria will be:

(i) inability to provide informed consent,
(ii) hemoglobin < 70 g/L,
(iii) Z scores for weight-for-age (WAZ) or weight-for-height (WHZ) <3,
(iv) any maternal or infant chronic illness,
(v) administration of any vitamin or mineral supplements for the past 2 months, and
(vi) any history of antibiotic treatment in the past 7 days.

**Intervention**: Each study group will receive in-home fortification for 6 months with multiple micronutrient powders with 5 mg iron (as ferrous fumarate [5.0 mg]) and

**Study group A**: Galacto-oligosaccharides, 7.5 mg.
**Study group B**: Bovine lactoferrin, 1.0 g.
**Study group C**: Galacto-oligosaccharides, 7.5 mg, and bovine lactoferrin, 1.0 g.
**Study group D**: No galacto-oligosaccharides or bovine lactoferrin (Placebo)

The multiple micronutrient powders are composed of Vitamin A, 400 **µ**g; Vitamin D, 5 **µ**g; Vitamin E, 5 mg; Thiamine, 0.5 mg; Riboflavin, 0.5 mg; Vitamin B6, 0.5 mg; Folic Acid, 90 **µ**g; Niacin, 6 mg; Vitamin B12, 0.9 **µ**g; Vitamin C, 30 mg; Copper, 0.56 mg; Iodine, 90 **µ**g; Selenium, 17 **µ**g; Zinc, 4.1 mg; Phytase, 190 FTU; Iron, 5 mg (as ferrous fumarate [5.0 mg]). In the intervention for Study Group D, the usual maltodextrin carrier will be added to a weight of 10.0 g. In Study Groups A and C, galacto-oligosaccharides will replace the maltodextrin and will be added as 10.0 g Vivinal GOS 75 Powder (Friesland Campina, Wageningen, The Netherlands) containing 7.5 g of galactooligosaccharides. This dose of 7.5 g/day was selected based on studies that reported a bifidogenic effect in infants and adults at doses from 2.5 to 10 g/day97 and the typical daily dose delivered by commercial infant formula containing galacto-oligosaccharides. In Study Groups B and C bovine lactoferrin (Friesland Campina, >90% apo-lactoferrin), 1.0 g will be added. The dose of lactoferrin was chosen based on the estimated amount consumed by a breastfeeding 12-month-old infant.88 The micronutrient powders will be packed in identical appearing group-coded sachets (each containing one daily dose) at the factory, and the codes will be kept solely by the Data and Safety Monitoring Board (DSMB) and the producer; study participants and investigators will be blinded to group assignment.

**Trial design and study procedures**: As shown in Figure 2, 288 infants will be recruited during the 5-year trial. After obtaining informed consent by either a written signature or a fingerprint from the caregiver, baseline screening will include collection of a 3 mL venous blood sample and measurement of infant weight and infant length. Participating caregivers will be carefully instructed in cooking the *uji* and its fortification with the micronutrient powders, and in the proper home collection of the infant stool samples. Before starting the intervention, a 2-week run-in period without micronutrient powders will be conducted to familiarize the families with preparation of the maize porridge and the method of stool collection. During this run-in period, a baseline fecal sample will be collected from the infants. Stratified randomization will be carried out by the Trial Statistician using computer-generated randomly permuted blocks of size 2, 4 or 6 with stratification by gender and assignment of eligible infants to one of the 4 intervention groups using 4 color codes. Allocation will be known solely by the Trial Statistician and the Clinical Trial Safety Officer and concealed from all other study personnel. Subsequently, for the next 6 months, weekly visits will be made to the homes of the participating infants. At each weekly visit for the first 6 months, we will dispense 7 multiple micronutrient powder sachets and 2 kg of refined maize flour (Mombasa Maize Millers Ltd, Mombasa, Kenya) to the caregivers for use in the preparation of all porridge fed to the infant. During weekly visits, we will collect the used and unused micronutrient powder sachets from the previous week to assess adherence. We will evaluate infant morbidity over the previous 7 days using the Weekly Morbidity Questionnaire containing forced-choice questions on fever, diarrhea, blood or mucus in the stool, cough, difficult or rapid breathing, and other illnesses. The Weekly Morbidity Questionnaire will be administered verbally and the responses recorded by the study team (Village Monitor). If a mother reports any current illness in the child, mother and child will be transported to the Msambweni District Hospital, examined and treated by the study clinicians, who will record treated episodes of malaria, diarrhea, respiratory tract infections, and other illnesses. Each infant will then be followed for an additional 3 months to determine the longer-term effects of the study treatments. During the last three months, the

frequency of home visits will be reduced from one every seven days to one every fourteen days. Identical procedures as in the first 6 months will be used to evaluate infant morbidity and the provision of MNPs will be stopped. At 1 month, 6 months and 9 months after study entry, we will collect a 3 mL venous blood sample, obtain a stool sample and measure length and weight. Any infants remaining anemic at 6 months will be examined and treated according to local guidelines.

**Data collection**: After obtaining venous blood samples at baseline, 1, 6 and 9 months, hemoglobin concentrations will be measured immediately using a HemoCue 300 analyzer, with daily measurement of controls. Plasma will be separated by centrifugation, frozen on the collection day and stored at -20°C until analysis. At ETH-Zürich, plasma ferritin, soluble transferrin receptor, C-reactive protein and alpha-1-acid glycoprotein will be analyzed using multiplex immunoassay. Intestinal fatty acid binding protein (I-FABP) will be measured by immunoassay. Blood samples will be frozen, stored at -20°C, and sent to Columbia University for genotyping for hemoglobin S and the common African form of $\alpha$-thalassemia caused by a 3.7-kilobase pair deletion in the $\alpha$-globin gene.

Stool samples will be collected at baseline and at 1, 6 and 9 months after the start of the intervention. Short-chain fatty acids (acetate, propionate, butyrate), branch chain fatty acids (BCFA), and intermediate products (lactate, formate, succinate) will measured at ETH-Zurich using high performance liquid chromatography (HPLC). Fecal calprotectin will be measured by an enzyme-linked immunosorbent assay. Two Kato-Katz thick smears will be prepared from each stool sample according to standard protocols[1] and helminth eggs counted microscopically. Fecal DNA extraction, gut microbiome analysis using 16S rRNA gene amplicon sequencing and targeted quantitative real-time polymerase chain reaction (qPCR) will be carried out as previously described. Targeted qPCR will be performed using specific primers for the bacterial subgroups most prevalent in the infant gut and expected low-abundance pathogens, as previously described.

**Sample size**: Based on the preliminary data from our study with galacto-oligosaccharides (GOS), a sample size of 288 subjects (72 in each group) would permit detection of a minimum standard deviation of size 0.2 among the standardized means of the 4 groups with 80% power at the 0.05 significance level. In the preliminary data from the GOS study, the standard deviation of the ratios of harmful pathogens and beneficial bacteria in the study group receiving iron alone and the group receiving iron + GOS ranged from 0.002 to 0.1, so our study, with a sample size of 288 subjects, has 80% power to detect a standard deviation of 0.0004 to 0.02.

**Primary outcome**: The primary outcome measure will be the ratio of the abundances of potentially harmful (enteropathogenic and/or enterotoxigenic *E. coli, C. difficile*, members of the *C. perfringens group, B. cereus, S. aureus*, sum of *Shigella* spp., and *Salmonella*) to beneficial (bifidobacteria and the group of La*ctobacillus/Leuconostoc/Pediococcus* spp.) bacterial genera at 1 month.

**Principal secondary outcomes**: The principal secondary outcome measures will include the ratio of the abundances of potentially harmful to beneficial bacterial genera ratios at 6 and 9 months and comparisons of microbiota composition at all time points, including assessment of effects of helminth infections. Other principal secondary outcomes will include the prevalence at 1, 6 and 9 months of diarrhea, malaria, anemia, iron deficiency, iron deficiency anemia, inflammation, respiratory tract infections, and other illnesses.

**Data Coordinating Center and Statistical analysis**:

Columbia University will serve as the Data Coordinating Center for the project. The descriptive statistics of baseline demographic and clinical characteristics of the study groups will be presented in tabular form. For each primary and secondary outcome, a summary of results for each study group with the estimated effect size and its precision will be reported. Data distributions will be checked for normality; data not normally distributed will be log transformed.

Data will be analyzed using IBM SPSS Statistics 24.0.0 (SPSS Inc., Chicago, IL) and Microsoft Office EXCEL 2016 (Microsoft, Redmond, WA). The primary statistical analysis will be by "intention to treat." Sequence data will be processed using QIIME, augmented by the R package QIIMER. Taxonomy will be assigned to the sequences using Ribosomal Database Project (RDP) for 16S, augmented by analysis of specific sequences using BLAST. The 16S tag sequences will be collected into operational taxonomic units (OTUs) with 97% sequence identity and samples summarized as vectors of proportions. Stool samples will be compared longitudinally within individuals. The influence of helminths will be assessed. The bacterial abundances at genus level will be quantified using QIIME. Low sequencing depth samples and low abundant genus will be removed. We will estimate the microbial diversity of a sample by calculating distance measures based on various metrics, including the Shannon diversity index ($\alpha$ diversity) and UniFrac distances ($\beta$ diversity). The primary outcome, as measured by the ratio of harmful and beneficial bacterial taxa, will be compared among the 4 treatment groups using repeated-measure ANOVA.

To test the associations between the study treatments (GOS, bLF, GOS+bLF, no GOS or bLF) and microbial abundance, we will use PERMANOVA based on UniFrac distance to test whether the overall microbial compositions differ among the four treatment groups at 1, 6 and 9 months after treatment. To deal with the excessive zeros observed in such data, we will use a two-part zero-inflated Beta regression model with random effects to examine differences in changes between baseline and the 1-, 6- and 9-month measurements for each of the bacterial genera. The Benjamini-Hochberg methods will be used to control for false discovery with multiple tests. The principal coordinate analysis (PCoA) based on phyla-level phylogeny or count-based distance metrics will also be used for group comparisons and for exploratory analysis. A number of secondary statistical analyses will be carried out. Analysis of group differences in change over time will use mixed linear model analysis with fixed effects for group, time, and group by time interaction; random effects for subject and error; and a compound symmetry covariance structure for within-subject correlation between times.

**Expected results:** We anticipate that the relative order of the ratio of the abundances of potentially harmful bacterial groups to beneficial bacterial groups will be significantly different between the study groups, ranging (from most to least protective) Study Group C (GOS + bLF) < Study Group B (bLF) < Study Group A (GOS) < Study Group D (no GOS, no bLF).

Specific Aim 2: to examine microbiota composition, metabolic activity, and inflammatory potential *in vitro* with treatments paralleling those in Specific Aim 1, using immobilized fecal microbiota from Kenyan infants to inoculate our long-term continuous polyfermenter intestinal model *in vitro* in conjunction with cellular studies. The fecal microbiota will be derived from de-identified stool samples from the Kenyan infants participating in the studies described in Specific Aim 1

Using our long-term continuous PolyFermS intestinal *in vitro* model to simulate conditions in the Kenyan infant proximal colon combined with cellular models, our primary hypothesis is that cultivating immobilized fecal microbiota from Kenyan infant donors in a medium mimicking the chime entering the colon with gGOS, bLF, the combination of GOS and bLF, and with nutritive medium alone, will result in a relative order of the ratio of the abundances of potentially harmful genera to beneficial bacterial and of gut microbiota inflammation potential of GOS + bLF < bLF < GOS < no GOS, no bLF.

## Risks to Human Subjects

**a. Human Subjects Involvement, Characteristics, and Design:** Because the overall goal of this research is to develop a means to safely administer iron supplements to infants in settings with a high infection burden, infants in costal Kenya will be recruited for this study. The clinical trial will be conducted in the catchment area of the Kikoneni health center in southern coastal Kenya, a malaria-endemic region with a high infectious disease burden, that is the site of our previous studies of micronutrient powders with iron and of our preliminary studies with galacto-oligosaccharides described in the research proposal. This region experiences a long rainy season from April to July, and short rains from October to November. Farming is the main economic activity and maize the staple food crop in this sparsely populated area. The population to be studied will consist of 288 infants, 6 months (± 3 weeks) of age with both infant and mother in good health, free of any acute or chronic illness. The study is a single-center, double-blinded, randomized, 9-month clinical trial with a 2X2 factorial design to determine the efficacy of galacto-oligosaccharides and bovine lactoferrin in preserving a beneficial gut microbiota during iron supplementation in Kenyan infants. Inclusion criteria will be (i) vaginal or cesarean delivery, (ii) an infant age of 6 months (±3 weeks), (iii) mother ≥15 years of age, (iv) infant still breastfeeding, and (v) anticipated residence in the area for the study duration. Exclusion criteria will be (i) inability to provide informed consent, (ii) hemoglobin < 70 g/L, (iii) Z scores for weight-for-age (WAZ) or weight-for-height (WHZ) <3, (iv) any maternal or infant chronic illness, (v) administration of any vitamin or mineral supplements for the past 2 months, and (vi) any history of antibiotic treatment.

Each study group will receive in-home fortification for 6 months with multiple micronutrient powders with 5 mg iron (as ferrous fumarate [5.0 mg]) and

**Study group A**: Galacto-oligosaccharides, 7.5 mg.

**Study group B**: Bovine lactoferrin, 1.0 g.

**Study group C**: Galacto-oligosaccharides, 7.5 mg, and bovine lactoferrin, 1.0 g.

**Study group D**: No galacto-oligosaccharides or bovine lactoferrin.

The study design does not include a group of infants given micronutrient powders without iron because of ethical considerations. Our previous studies in this population have shown that no decrease in anemia or improvement in hemoglobin concentration is found in groups of infants given micronutrient powders without iron. The multiple micronutrient powders are composed of Vitamin A, 400 µg; Vitamin D, 5 µg; Tocopherol Equivalents, 5 mg; Thiamine, 0.5 mg; Riboflavin, 0.5 mg; Vitamin B6, 0.5 mg; Folic Acid, 90 µg; Niacin, 6 mg; Vitamin B12, 0.9 µg; Vitamin C, 30 mg; Copper, 0.56 mg; Iodine, 90 µg; Selenium, 17 µg; Zinc, 4.1 mg; Phytase, 190 FTU; Iron, 5 mg as ferrous fumarate [5.0 mg]). In the intervention for Study Group D, maltodextrin will be added to a weight of 11.0 g. In Study Groups A and C, galacto-oligosaccharides will be added as Vivinal GOS 75 Powder (Friesland Campina, Wageningen, The Netherlands This dose of 7.5 g/day was selected based on studies that reported a bifidogenic effect in infants and adults at doses from 2.5 to 10 g/day and the typical daily dose delivered by commercial infant formula containing GOS. In Study Groups B and C bLF (Friesland Campina, >90% apo-lactoferrin), 1.0 g will be added. The dose of bLF was chosen based on the amount used in a previous clinical trial that showed a decrease in the severity and longitudinal prevalence of diarrhea. The micronutrient powders will be packed in identical appearing group-coded sachets (containing one daily dose) at the factory, and the codes will kept solely by the Data and Safety Monitoring Board (DSMB) and the producer; study participants and investigators will be blinded to group assignment. Both galacto-oligosaccharides and bovine lactoferrin are classified as Generally

Recognized As Safe (GRAS) by the U.S. Food and Drug Administration, are components of cow's milk and have been used repeatedly in clinical trials without adverse effects.

**b. Sources of Materials:** After obtaining informed consent by either a written signature or a fingerprint from the caregiver, baseline screening will include collection of a 3 mL venous blood sample and measurement of infant weight using a Salter-type baby weighing scale to the nearest 0.1 kg and of infant length using a rigid measurement board to the nearest 0.5 cm. Before starting the intervention, a 2-week run-in period without micronutrient powders will be conducted to familiarize the families with preparation of the maize porridge and the method of stool collection. During this run-in period, a baseline fecal sample will be collected from the infants. Subsequently, for the next 9 months, weekly visits will be made to the homes of the participating infants. At each weekly visit for the first 6 months, we will dispense 7 multiple micronutrient powder sachets and 2 kg of fortified, refined maize flour (Mombasa Maize Millers Ltd, Mombasa, Kenya) to the caregivers for use in the preparation of all porridge fed to the infant. During weekly visits, we will encourage adherence to the study protocol and evaluate infant morbidity over the previous 7 days using a questionnaire. If a mother reports any current illness in the child, mother and child will be transported to the Msambweni District Hospital, examined and treated by the study clinicians, who will record treated episodes of malaria, diarrhea, respiratory tract infections, and other illnesses. Using identical procedures but for stopping provision of micronutrient powders, each infant will then be followed for an additional 3 months to determine the longer-term effects of the study treatments. At 1 month, 6 months and 9 months after study entry, we will collect a 3 mL venous blood sample, obtain a stool sample and measure length and weight as at the baseline examination. Any infants remaining anemic at 6 months will be examined and treated by the study clinicians according to local guidelines.

The Principal Investigator will be responsible for the accumulation and maintenance of appropriate data files and the preservation of the confidentiality and security of these files. The need for strict confidentiality of all study records will be emphasized to the staff of the study. Subjects will not be identified by name on any study documents and will be identified solely by a Study Identification Number. All reports and summaries prepared by the investigators will be presented in such a way that no individual participant can be identified. The data collected in this study will remain under the control of the Principal Investigator and will be kept confidential with the sole exception that Hospital or Government authorities may have access to records containing the identity of study volunteers. If publications result, volunteer names will not be used. All forms will be kept in locked cabinets. All computerized files will be encrypted and protected by a password system.

No individually identifiable private information will be collected specifically for the proposed research project. For supervision of all research material for this study, the Principal Investigator, with the assistance of the Research Staff, will be responsible for data management, including data collection from Case Report Forms, and for the review, entry, editing, reduction, analysis and display of data with the generation of summary reports. Procedures for specimen identification and handling will be established. Study personnel will be familiarized with data flow and procedures for data security, verification and error checking. In all phases of data editing, an audit trail will be developed to permit identification of all changes made in the data file and the dates of these changes; thus it will be possible to reconstruct the database at any point in time.

**c. Potential Risks:** When blood is drawn from a vein in the arm, there may be some temporary discomfort and the minimal risk of local bruising, infection or blockage of the vein. Suitable precautions will be taken to avoid these risks. Both galacto-oligosaccharides and bovine lactoferrin are classified as Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration, are components of cow's milk and have been used repeatedly in clinical trials

without adverse effects. In the absence of adequate health care, provision of supplemental doses of iron (≥12.5 mg Fe/day; greater than the dose to be used in this study) may increase the risk for malaria and may modify the infant gut microbiota resulting in diarrhea.

**Adequacy of Protection Against Risks**

**a. Recruitment and Informed Consent:** Potential participants will be recruited from mothers and infants attending well-child clinics at the Msambweni District Hospital, Kenya, by study staff trained in recruitment procedures, who will fully describe the study, using the approved Participant Consent Form as a template. Only mothers with breastfeeding infants will be offered enrollment in the study. Detailed oral and written information explaining the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits will be provided to interested mothers. After sufficient time for decision (24 hours) has been given, written informed consent will be sought. Oral and written information, as well as the informed consent form, will be available in English and Swahili, the two official languages in Kenya. If the caregiver(s) are illiterate, the informed consent will be read out by an independent witness (e.g. personnel from the Msambweni District Hospital).

**b. Protections Against Risk:** Suitable precautions will be taken to avoid the minimal risks of venipuncture**.** The low dose of iron (5.0 mg Fe/day) to be used in the present study, along with giving the iron with complementary foods, will minimize any infectious risks. Morbidity of the infants will be closely monitored and infectious diseases, such as malaria and diarrhea, will be promptly treated according to Kenyan national guidelines.

**c. Potential Benefits of the Proposed Research to Human Subjects and Others:** The benefits of the study include the distribution of micronutrient powders with iron that will almost certainly improve the micronutrient status of all study participants. In addition, the health of each infant will be closely monitored throughout the study and any illness will be treated without charge at the Msambweni District Hospital. Additionally, a training program in mother-child nutrition will be offered to the mothers of study participants including instructions on child feeding, complementary foods, food preparation, and general parenting skills. These benefits clearly would seem to outweigh the minimal risks to study participants.

**d. Importance of the Knowledge to be Gained:** The overall goal of this research is to develop a means to safely administer iron supplements to infants in settings with a high infection burden. The knowledge to be gained in our study will help to guide formulation of safer iron supplements and fortificants and to identify the mechanisms whereby prebiotic galacto-oligosaccharides and bovine lactoferrin support the commensal microbiota to prevent iron-induced overgrowth by opportunistic enteropathogens. The results of our study could help remove a major obstacle to the safe supply of supplemental iron to infants and children in resource-limited settings, a global public health problem.

# Data Safety Monitoring Plan

## I. Overview

**A. Brief description of the purpose of the study**. The overall purpose of this research is to develop a means to safely administer iron supplements to infants in settings with a high infection burden. The study will determine if promoting development of a beneficial, protective gut microbiota by co-administration of prebiotic galacto-oligosaccharides (GOS) and bovine lactoferrin (bLF) during iron supplementation will prevent iron-induced increases of opportunistic enteropathogens that cause infection and inflammation.

**B. Adherence statement**. The Data and Safety Monitoring Plan (DSMP) outlined below for the proposed study will adhere to the protocol approved by the Institutional Review Boards (IRB) of the Columbia University Medical Center, the Swiss Federal Institute of Technology (Eidgenoessische Technische Hochschule - ETH Zürich), and the Jomo Kenyatta University of Agriculture and Technology.

## II. Adverse Events

### A. Adverse event assessment

1. The only expected risks with this study are the minimal risks of venipuncture and diarrhea. Episodes of malaria, diarrhea, respiratory tract infections, and other illnesses will be tracked weekly and recorded on study questionnaires for each participant. These risks are addressed in the protocol and consent form. If a mother reports any current illness in the child, mother and child will be transported to the Msambweni District Hospital, examined and treated by the study clinicians in accordance with Kenyan national guidelines. Treated episodes of malaria, diarrhea, respiratory tract infections, and other illnesses will be recorded.

2. Each subject will be evaluated for adverse events by weekly visits by study personnel.

3. Assessment of adverse events: For the purposes of this study, an Adverse Effect is defined as an undesirable event or experience occurring to a study subject during participation in the clinical trial. Any Adverse Event or Effect that is reported to the designated Research Staff by the mother of a participant or by the medical staff caring for the participant will be documented as such. All Adverse Effects will be graded as to their attribution (unrelated to the clinical trial protocol, or possibly, probably, or definitely related to the clinical trial protocol). A Serious Adverse Effect is defined as an Adverse Effect that is fatal, life-threatening, disabling or which results in the patient being hospitalized or, if already in the hospital, in hospitalization being prolonged. Any study-related Serious Adverse Effect will be reported within 24 hours to the Principal Investigator at the Jomo Kenyatta University of Agriculture and Technology, who will have responsibility for reporting the Serious Adverse Event to the Principal Investigators at the Swiss Federal Institute of Technology (ETH Zürich) and the Columbia University Medical Center, who will have the additional responsibilities of reporting the Serious Adverse Event to the Clinical Trial Data and Safety Monitoring Board and the ETH Zurich and Columbia University Institutional Review Boards. Medical follow-up of any Serious Adverse Event will be provided by the medical staff of the Msambweni District Hospital, in hospital or, if needed, in the out-patient clinic at appropriate intervals until resolution of the condition related to the Serious Adverse Event. An Adverse Event that does not meet the criteria of a Serious Adverse Event will be reported promptly to the Principal Investigators, appropriate management will be provided by the medical staff of the Msambweni District Hospital and the occurrence of any Adverse Events reported within one week to the Clinical Trial Data and Safety Monitoring Board, the ETH Zürich and Columbia University Institutional Review Boards, and the NIDDK Project Officer.

**B. Adverse event reporting**

1. Every adverse event that is reported to either the designated research staff by the mother of a study participant or by the medical staff caring for the subject that meets the criteria above will be documented.

2. An adverse event report will be generated for each event and will include a description of the event, the time of the event, the method of reporting, and any official chart records or documentation to corroborate the event, along with determination of attribution by the attending medical staff.

3. Recipients and timeline for adverse event reporting. Any study-related Serious Adverse Effect will be reported within 24 hours to the Principal Investigator at the Jomo Kenyatta University of Agriculture and Technology, who will have responsibility for promptly reporting the Serious Adverse Event to the Principal Investigators at the Swiss Federal Institute of Technology (ETH Zürich) and the Columbia University Medical Center, who will have the additional responsibilities of reporting the Serious Adverse Event to the Clinical Trial Data and Safety Monitoring Board and the ETH Zurich and Columbia University Institutional Review Boards and the NIDDK Project Officer. Medical follow-up of any Serious Adverse Event will be provided by the medical staff of the Columbia University Medical Center in hospital or, if needed, in the out-patient clinic at appropriate intervals until resolution of the condition related to the Serious Adverse Event. An Adverse Event that does not meet the criteria of a Serious Adverse Event will be reported promptly to the Principal Investigators, appropriate management will be provided by the medical staff of the Msambweni District Hospital and the occurrence of any Adverse Events reported within one week to the Clinical Trial Data and Safety Monitoring Board, the ETH Zürich and Columbia University Institutional Review Boards, and the NIDDK Project Officer.

4. Any action resulting in a temporary or permanent suspension of this study, either by the investigators, the Clinical Trial Data and Safety Monitoring Board, or by the ETH Zürich and Columbia University Institutional Review Boards, will be immediately reported to the NIDDK Program Officer for the study.

**III. Safety Review Plan and Monitoring**

**A. Justification of sample size**: Based on the preliminary data from our study with galacto-oligosaccharides (GOS), a sample size of 288 subjects (72 in each group) would permit detection of a minimum standard deviation of size 0.2 among the standardized means of the 4 groups with 80% power at the 0.05 significance level. In the preliminary data from the GOS study, the standard deviation of the ratios of harmful pathogens and beneficial bacteria in the study group receiving iron alone and the group receiving iron + GOS ranged from 0.002 to 0.1, so our study, with a sample size of 288 subjects, has 80% power to detect a standard deviation of 0.0004 to 0.02.

**B. Safety and study progress reviews**:

1. To maintain blinding of participant treatment assignment during safety monitoring, a Clinical Trial Safety Officer, a physician expert in Clinical Pediatrics, will be appointed to review any reports of adverse events and any laboratory results as they are reported during the course of the clinical trial to detect any abnormality requiring intervention. Abnormalities that require any intervention will be reported immediately to the Principal Investigators and summarized for annual reports to the Clinical Trial Data and Safety Monitoring Board, the ETH Zürich and Columbia University Institutional Review Boards, and the NIDDK Project Officer.

2. The Principal Investigators will review reports of recruitment, retention, and protocol adherence quarterly and the Clinical Trial Data and Safety Monitoring Board will review summary reports twice yearly.

3. The annual report for the Clinical Trial will include (i) a list and summary of adverse events and whether these exceed the minimal rate anticipated, (ii) a summary of recruitment and retention and reason for study dropouts, and (iii) an assessment of trial progress toward achieving the specific aims of the study.

4. Stopping Rules: Because this is a minimal risk study, the trial protocol does not stipulate stopping rules.

**IV. Informed Consent:** Potential participants will be recruited from mothers and infants attending well-child clinics at the Msambweni District Hospital, Kenya. Study staff trained in recruitment procedures will fully describe the study, using the approved Participant Consent Form as a template. Only mothers with breastfeeding infants will be offered enrollment in the study. Detailed oral and written information explaining the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits will be provided to interested mothers. After sufficient time for decision (24 hours) has been given, written informed consent will be sought. Oral and written information, as well as the informed consent form, will be available in English and Swahili, the two official languages in Kenya. If the caregiver(s) are illiterate, the informed consent will be read to the mother by an independent witness (e.g. personnel from the Msambweni District Hospital).

**V. Data Quality and Management**

**A**. The Principal Investigator will be responsible for the accumulation, maintenance and quarterly review of appropriate data files and of protocol adherence. For supervision of all research material for this study, the Principal Investigator, with the assistance of the Research Staff, will be responsible for data management, including data collection from Case Report Forms, and for the review, entry, editing, reduction, analysis and display of data with the generation of summary reports. Procedures for specimen identification and handling will be established. Study personnel will be familiarized with data flow and procedures for data security, verification and error checking. In all phases of data editing, an audit trail will be developed to permit identification of all changes made in the data file and the dates of these changes; thus it will be possible to reconstruct the database at any point in time.

**B**. The Principal Investigator will be responsible for the integrity, security and preservation of all study files. The data collected in this study will remain under the control of the Principal Investigator with the sole exception that Hospital or Government authorities may have access to records. If publications result, volunteer names will not be used. All forms will be kept in locked cabinets. All computerized files will be encrypted and protected by a password system. In all phases of data editing, an audit trail will be developed to permit identification of all changes made in the data file and the dates of these changes; thus it will be possible to reconstruct the database at any point in time.

**VI. Confidentiality: T**he Principal Investigator will be responsible for the preservation of the confidentiality of all study files. The need for strict confidentiality of all study records will be emphasized to the staff of the study. Subjects will not be identified by name on any study documents and will be identified solely by a Study Identification Number. All reports and summaries prepared by the investigators will be presented in such a way that no individual participant can be identified. The data collected in this study will remain under the control of the Principal Investigator and will be kept confidential with the sole exception that Hospital or

Government authorities may have access to records containing the identity of study volunteers. If publications result, volunteer names will not be used. All forms will be kept in locked cabinets.

**VII. Data Safety Monitoring Board:** A Clinical Trial Data and Safety Monitoring Board (DSMB) will be organized, consisting of a Chair with experience in the oversight of pediatric clinical trials, a biostatistician, an expert in tropical clinical pediatrics, a nutritionist, and an investigator with experience in analysis of microbiome data. The primary responsibility of the DSMB will be to act in an advisory capacity to the Principal Investigators to safeguard the interests of trial participants by monitoring participant safety, assess participants risk versus benefit, and assess data quality and general evaluation of the trial progress. The activity of the DSMB will delineated in a charter that will define the membership, responsibilities and the scope and frequency of data reviews. The DSMB will operate on a conflict-free basis independently of the PI and the study team.

The DSMB will have an organizational meeting prior to commencement of the trial and then twice yearly for the duration of the clinical trial. The Principal Investigators or the DSMB may convene *ad hoc* meetings if safety concerns arise during the trial. After assessment at each meeting, the DSMB will recommend to the Principal Investigators continuation, modification or termination of the study.