Official Title: Dietary Study of Human Milk Oligosaccharides with *Bifidobacterium infantis* in Antibiotic-treated Healthy Adult Volunteers

Unique Protocol ID: 21-CT-003

NCT ID: NCT05141903

Document Date: 15 June 2023

STATISTICAL ANALYSIS PLAN

Protocol: 21-CT-003

Dietary Study of Human Milk Oligosaccharides with Bifidobacterium infantis in Antibiotic-treated Healthy Adult Volunteers

SAP Version: 1.0 **Date of Final Change: 15 June 2023**

Date	Prepared by: Sheryl O'Rourke, MS, Consultant
Date	Approved by: Martin Lee, PhD, CStat, CSci.. Vice-President Clinical Research and Development
Date	Approved by: David Rechtman, MD. Vice-President, Medical Affairs

Published Date: 13Jun23

Revision History

Version #	Date Effective	Page/Section/ Paragraph	Change From:	Change To:

TABLE OF CONTENTS

1 LIST OF ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviation	Definition
AE	Adverse event
ALT	Alanine transaminase
AST	Aspartate transaminase
BUN	Blood urea nitrogen
BMI	Body-mass index
CRF	Case report form
CRO	Clinical research organization
DSMB	Data and safety monitoring board
EIA	Enzyme immunoassay
FDA	Food and Drug Administration
GRAS	Generally regarded as safe
GDH	Glutamate dehydrogenase
GCP	Good clinical practice
HIPAA	Health Insurance Portability and Accountability Act
HMO	Human milk oligosaccharide
ID	Identification number
IEC	Independent ethics committee
ISDA	Infectious Disease Society of America
IRB	Institutional review board
ICH	International Conference on Harmonization
NDA	Investigational New Drug
LDH	Lactate dehydrogenase
LNnT	Lacto-N-neotetraose
NGS	Next-generation sequencing

Abbreviation	Definition
NAAT	Nucleic acid amplification tests
OTU	Operational taxonomic unit
OTC	Over the counter
PCR	Polymerase chain reaction
PO	Per os
RTF	Ready-to-feed
SAE	Serious adverse event
SHEA	Society of Hospital Epidemiologists of America

2 INTRODUCTION

2.1 PREFACE

Human milk oligosaccharide (HMO) have recently become the objects of scientific inquiry. These complex sugars are extremely heat stable and are therefore found intact in donor milk and human milk-based products following pasteurization as well as in untreated mother's own milk.[1,2,4] Approximately 200 different HMO structures have been identified, although any particular mother will only produce a subset of those. Studies have shown that HMOs can alter the gut microbiome by favoring the growth of certain organisms as well as, perhaps, by inhibiting others.[4] It has also been demonstrated that HMOs can act as "decoy" receptors for a variety of pathogens and toxins.[4] They have also been shown, in vitro, to directly interact with certain components of the adaptive immune system.[4]

As a result of the aforementioned activities, it is reasonable to expect that these molecules may be useful as therapeutic agents or medical foods. This is particularly the case in medical settings where the HMOs themselves or their metabolites may provide missing nutrients or other metabolic products for the dietary management of diseases where the microbiome plays a role, either in whole or in part. Examples might include conditions as disparate as inflammatory bowel disease and stem cell transplantation.

We hypothesize that *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) might be a particularly beneficial organism to expand in the microbiome when managing such diseases in combination with HMOs. In infants, co-administration of *B. infantis* with human milk (via breast feeding) results in expansion of *B. infantis*, reduction in other potentially deleterious species, notably members of the Family Enterobacteriaceae, and acidification of baby's stool through the production of certain short chain fatty acids.[7] These dietary effects are postulated to be due to metabolism of HMOs by *B.*

infantis, as infants receiving human milk in the absence of *B. infantis* don't show these effects. Importantly, *B. infantis* is not observed as a constituent of the human adult gut microbiota so it may therefore be necessary to administer both HMOs and *B. infantis* to obtain the full beneficial impact of HMOs feeding in adults.

Prolacta Bioscience manufactures human milk-based products from donated breast milk. In the course of the manufacture of human milk-based human milk fortifier, skim breast milk is ultra-filtered in order to concentrate proteins. Investigation of the permeate produced through this process demonstrated that human milk oligosaccharides were present in amounts comparable to those found in raw breast milk. Moreover, as the starting material consists of breast milk pooled from between 100 and 200 mothers, the full range of HMOs could be found. Subsequently, a method was developed to allow for the production of material with elevated concentrations of HMOs which contains little to no residual lactose through the introduction of lactase which is then removed.

The HMO concentrate PBCLN-010 is a frozen liquid 15% concentration of HMO derived from the permeate obtained in concentrating donor human milk during the manufacturing of Prolacta's human milk fortifiers. Previous versions of this product at 5% and 10% concentrations have been studied as a food supplement in healthy volunteers, either alone or in combination with B. infantis. A previous version has also been studied alone in C. difficile patients. No serious product-related adverse events have been reported in the course of these studies.

The synthetic HMO 2'FL + LNnT product in this trial is a powder containing 2'-fucosyllactose (2'-FL) and lactoneotetraose (LNnT). These HMO were also present in Prolacta's previous HMO studies, where donor-derived HMO concentrates were administered to subjects with no adverse events recorded. Here, they are present in a more purified mixture without the other HMO found in material used in the earlier trials. This HMO product is commercially available as a medical food in the US.

The *B. infantis* preparation being used in this trial is a dietary supplement commercially available in the United States and marketed for use in infants. Strains of *B. infantis* have been characterized as "generally regarded as safe" (GRAS) (Duar, 2020).

2.2 THE PURPOSE OF THE ANALYSES

Prolacta Bioscience recently completed a study in healthy volunteers which demonstrated that a concentrate of HMO derived from human milk can drive *B. infantis* colonization in some healthy volunteers (20-CT-001, 2020). The purpose of this study, 21-CT-003, is three-fold. One goal is to determine if the rate of HMO-supported colonization with *B. infantis* is increased if the microbiome of the volunteers has been disrupted by administration of antibiotics. The second goal is to determine whether microbiome recovery post-antibiotic treatment can be improved by the presence of *B. infantis*. The third goal is to determine the mechanism of HMO-supported

colonization; whether a selected subset of HMO (2'FL + LNnT) can support *B. infantis* colonization to the same extent as the full complement of HMO present in the HMO concentrate used previously.

2.3 STUDY ENDPOINTS

2.3.1 Primary

The primary endpoint will be changes in the level of *B. infantis* from baseline evaluated by quantitative PCR using primers specific for the subspecies. Quantitation limits will be demonstrated by qualification assays demonstrating the lower limit of detection, in order to establish minimum log-fold change in *B. infantis*, as it is expected that adult subjects will not harbor B. infantis in baseline samples.

2.3.2 Secondary

- Changes in stool microbiota

Stool microbiota changes will be measured as well as dynamic changes in the gut community structure. These changes will be evaluated by next generation sequencing using proportions of key bacterial operational taxonomic units (OTUs), relative abundance of various taxa, diversity (alpha and beta) and functional metabolomic changes. Genera- and family-specific primers will also be used to perform qPCR to gauge changes in the microbial community structure.

- Changes in viability of Proteobacteria and Enterococcus sp.

These will be determined by plating on selective media. These measurements will be predictive for success in suppressing pathogenic bacterial relatives in future trials.

- Changes in the carriage load of antibiotic resistance genes

Whole metagenomic sequencing (as described above) will allow characterization of functional genes found in the bacterial taxon identified in each dose group. We will identify antibiotic resistance genes by aligning metagenomic sequences against a database that includes known antibiotic resistance genes.

- Blood parameters

Blood parameters, including cytokine levels, will be evaluated at the screening visit and on study days 1, 3, 5, 9, 14, 28 and 35. Serum will also be assessed for metabolite levels, including but not limited to HMO, short chain fatty acids, and indole.

- Secretor status will be determined by analyzing DNA collected via a cheek swab

2.3.3 Safety

- Total exposure to all study products will be presented in a listing.
- Adverse events will be summarized by severity and relationship to study product.
- The laboratory evaluations listed below will be evaluated for clinical lab and medical safety at the following visits: Screening, Day 1, Day 3, Day 5, Day 9, Day 14, Day 28 and Day 35.
    - CBC with differential and platelets
    - Alkaline phosphatase, alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), total and conjugated bilirubin, albumin and total protein for liver function
    - Electrolytes (Na, K, Cl, and HCO_3)
    - Glucose, total calcium, magnesium, phosphate, creatinine, and blood urea nitrogen (BUN) for renal function.
- Vital signs (heart rate, respiratory rate, temperature, and blood pressure are collected at the following visits: Screening, Day 1, and Day 35.
    - Heart rate
    - Respiratory rate
    - Temperature
    - Blood pressure

3 STUDY METHODS

3.1 STUDY DESIGN

We propose to conduct an unblinded five dosing group trial using 18 subjects per dosing group for a total of 90 healthy adult volunteers ages 18-75. The randomization scheme for this study is as follows. The first twenty subjects will be randomized into Dosing Group 1 or 2 using a randomized permuted block scheme. The next thirty four subjects will be randomized into Dosing Group 1, 2, or 3 using a similar separate randomized permuted block scheme employing a 4:4:9 ratio (group 1:group 2:group 3) in a block of 17. The remaining thirty six subjects will be randomized into Dosing Groups 4 or 5 using a separate but similar randomized permuted block approach employing a 1:1 ratio (group 4:group 5). The IP and medications for this study include: antibiotics, HMO PBCLN-010, HMO 2'FL + LNnT, and B. infantis.

Antibiotics will be given to all subjects in this study: vancomycin (250 mg/dose) and metronidazole (500 mg/dose).

The dosing regime is outlined as follows, refer to Table 1 additionally.

Subjects randomized to Dosing Group 1 will consume antibiotics three times daily (TID) for 5 days. Subjects will be followed from the first dose of the antibiotics (day 1) until day 35.

Subjects randomized to Dosing Group 2 will consume antibiotics three times daily (TID) for 5 days, HMO PBCLN-010 twice daily for 28 days and B. infantis once daily for 14 days. They will be followed from the first dose of the IP (day 1) until day 35.

Subjects randomized to Dosing Group 3 will consume antibiotics three times daily (TID) for 5 days and B. infantis once daily for 14 days. They will be followed from the first dose of the IP (day 1) until day 35.

Subjects randomized to Dosing Group 4 will consume antibiotics three times daily (TID) for 5 days, HMO PBCLN-010 twice daily for 9 days, B. infantis once daily for 14 days, and HMO 2'FL + LNnT twice daily for 19 days (to begin after HMO PBCLN-010 dosing has ended). They will be followed from the first dose of the IP (day 1) until day 35.

Subjects randomized to Dosing Group 5 will consume antibiotics three times daily (TID) for 5 days, B. infantis once daily for 14 days, and HMO 2'FL + LNnT twice daily for 28 days. They will be followed from the first dose of the IP (day 1) until day 35.

Table 1 below summarizes the dosing for each group.

Table 1 Dosing Cohorts

Dosing Group	**vancomycin** (250 mg/dose) and **metronidazole** (500 mg/dose) **(1 tablet of each antibiotic three times per day**	***B. infantis* (1 sachet once per day)**	**Daily target=18g HMO PBCLN-010 (9g twice per day)**	**Daily target=22g HMO 2'FL + LNnT (2 sachets twice per day)**
1	D1 – D5	None	None	None
2	D1 – D5	D1 – D14	D1 – D28	None
3	D1 – D5	D1 – D14	None	None
4	D1 – D5	D1 – D14	D1 – D9	D10 – D28
5	D1 – D5	D1 – D14	None	D1 – D28

3.2 INCLUSION/EXCLUSION CRITERIA AND THE GENERAL PATIENT POPULATION

3.2.1 Inclusion Criteria

- Healthy adults between the ages of 18-75 years (subjects must be 18-75 at the time of consent) who can provide proof of vaccination against SARS-CoV-2. Proof may be a physical or electronic record of vaccination or self-attestation (to include approximate vaccination date and manufacturer of vaccine) if a copy of the vaccination record is not available
- Subjects must have a BMI of 18 - 30 at screening visit
- Willingness to complete study specific questionnaires
- Willingness to complete journal to record IP dosing times, Bristol stool scores, and IP flavor questionnaires
- Willingness to complete all study procedures and clinic visits, and provide required biospecimen samples
- Able to provide stool samples on specific study days
- Willingness to collect and process stool samples at home and transport stool samples to clinic
- Sexually active females of child-bearing potential must agree to use highly effective methods of contraception during heterosexual intercourse throughout the study period and for three days following discontinuation of IP, whichever comes later. Examples of highly effective methods include the use of two forms of contraception with one being an effective barrier method (e.g., a condom and spermicide used together), or have a vasectomized partner. Abstinence is acceptable as a life-style choice. Female subjects who utilize hormonal contraceptives as one of their birth control methods must have used the same method for at least 3 months before study dosing
- Provides informed consent

3.2.2 Exclusion Criteria

- Subjects with a BMI of 17 or less or 31 or greater are excluded
- Women who are pregnant or breastfeeding, or intend to become pregnant during the course of this study
- Subjects who intend to take a probiotic during the study
- Subjects with self-reported diarrhea on day 1 prior to dosing, whereby diarrhea is defined as two or more episodes of watery and/or unformed stool within 24 hours

- Alcohol or drug abuse during the last 12 months, including passing a screen for drugs of abuse at screening and day 1 of the study
- Unstable medical condition, in the opinion of the investigator
- Subject with a history of allergy to vancomycin and/or metronidazole
- Clinically significant abnormal laboratory test results at screening
- Subjects who are unable or unwilling to provide stool samples on a regular basis as per study protocol
- Participation in a clinical research trial within 30 days prior to screening
- Unable to give informed consent
- Any condition which may preclude subject's ability to comply with and complete the study or may pose a risk to the health of the subject
- Known carriers of *C. difficile* prior to study start, as determined by qPCR of stool
- Known carriers of vancomycin-resistant enterococci (VRE) prior to study start, as determined by stool culture
- Subjects with history of lactose intolerance

3.3 RANDOMIZATION

The randomization scheme for this study is as follows. The first twenty subjects will be randomized into Dosing Group 1 or 2 using a randomized permuted block scheme. The next thirty four subjects will be randomized into Dosing Group 1, 2, or 3 using a similar separate randomized permuted block scheme employing a 4:4:9 ratio (group 1:group 2:group 3) in a block of 17. The remaining thirty six subjects will be randomized into Dosing Groups 4 or 5 using a separate but similar randomized permuted block approach employing a 1:1 ratio (group 4:group 5).

3.4 SCHEDULE OF EVENTS

The tables below give the schedule of visits and evaluations for each dosing group.

DOSING GROUP 1 SCHEDULE OF EVENTS Days 1 – 14

Events	Screening	Dosing Days					Washout Period					
	D0 (≤ 90 days prior to D1)	D1	D2	D3	D4	D5	D6	D7 (+1D)	D8	D9	D10	D11
Clinic visit	X	X		X		X				X		
Final Visit												
Informed consent	X											
Review IE Criteria	X	X										
Medical History	X											
Physical Exam	X											
Demographics (age, gender, ethnicity and	X											
Vital Signs (Temp, BP, pulse, RR)	X	X										
Height, weight and BMI	X											
Prior & Con Meds	X	X										
AEs		X		X		X				X		
Randomization		X										
Vancomycin 250 mg + metronidazole 500 mg (Q8hrs +/- 3 hours)		X	X	X	X	X						
Assess IP and stool compliance		X	X	X	X	X						
Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire		X	X	X	X	X	X	X	X	X	X	X
Patient phone calls		X	X	X	X	X						
Urine drug screen test	X	X										
Urine pregnancy test (females of child-bearing potential)	X	X										
Frozen urine aliquots (central lab – Indoxyl Sulfate)		X		X		X						
Hematology and chemistry testing	X	X		X		X				X		

Serum aliquots	X	X		X		X				X		
C-diff and VRE stool screening test	X											
Stool aliquots (no preservatives)	X	X		X		X				X		
Stool aliquots (with ethanol)					X			X				X
Cheek swab for secretor status		X										

DOSING GROUP 1 SCHEDULE OF EVENTS Days 15 – 35

Events	**Washout Period**											
	D15	D16	D17	D18	D19	D20	D21 (+ 2D)	D22	D23	D24	D25	D26
Clinic visit												
Final Visit												
Physical Exam												
Vital Signs (Temp, BP, pulse, RR)												
Prior & Con Meds												
AEs												
Assess IP and stool compliance												
Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire	X	X	X	X	X	X	X	X	X	X	X	X
Patient phone calls												
Frozen urine aliquots (central lab – Indoxyl Sulfate)												
Hematology and chemistry testing												
Serum aliquots												

Stool aliquots (no preservatives)												
Stool aliquots (with ethanol)			X				X					

DOSING GROUP 2 SCHEDULE OF EVENTS Days 1 – 14

Events	Screening	Dosing Days										
	D0 (≤ 90 days prior to D1)	D1	D2	D3	D4	D5	D6	D7 (+1D)	D8	D9	D10	D11 (+1D)
Clinic visit	X	X		X		X				X		
Final Visit												
Informed consent	X											
Review IE Criteria	X	X										
Medical History	X											
Physical Exam	X											
Demographics (age, gender, ethnicity and	X											
Vital Signs (Temp, BP, pulse, RR)	X	X							X			
Height, weight and BMI	X											
Prior & Con Meds	X	X							X			
AEs		X		X		X				X		
Randomization		X										
Vancomycin 250 mg + metronidazole 500 mg (Q8hrs +/- 3 hours)		X	X	X	X	X						
B. Infantis (QD)		X	X	X	X	X	X	X	X	X	X	X
HMO PBCLN-010 (BID)		X	X	X	X	X	X	X	X	X	X	X
Assess IP and stool compliance		X	X	X	X	X						
Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire		X	X	X	X	X	X	X	X	X	X	X
Patient phone calls		X	X	X	X	X	X	X	X	X	X	X
Urine drug screen test	X	X										

Urine pregnancy test (females of child-bearing potential)	X	X										
Frozen urine aliquots (central lab – Indoxyl Sulfate)		X		X		X						
Hematology and chemistry testing	X	X		X		X				X		
Serum aliquots	X	X		X		X				X		
C-diff and VRE stool screening test	X											
Stool aliquots (no preservatives)	X	X		X		X				X		
Stool aliquots (with ethanol)					X			X				X
Cheek swab for secretor status		X										

Dosing Group 2 Schedule of Events Days 15 – 35

Events	Dosing Days											
	D15	D16	D17	D18	D19	D20	D21 (+ 2D)	D22	D23	D24	D25	D26
Clinic visit												
Final Visit												
Physical Exam												
Vital Signs (Temp, BP, pulse, RR)												
Prior & Con Meds												
AEs												
HMO PBCLN-010 (BID)	X	X	X	X	X	X	X	X	X	X	X	X
Assess IP and stool compliance												
Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire	X	X	X	X	X	X	X	X	X	X	X	X
Patient phone calls	X	X	X	X	X	X	X	X	X	X	X	X
Frozen urine aliquots (central lab – Indoxyl Sulfate)												
Hematology and chemistry testing												

Serum aliquots												
Stool aliquots (no preservatives)												
Stool aliquots (with ethanol)			X				X					

Dosing Group 3 Schedule of Events

Events	**Screening**	**Dosing Days**										
	D0 (≤ 90 days prior to D1)	D1	D2	D3	D4	D5	D6	D7 (+1D)	D8	D9 (+1D)	D10	D1 (+2D
Clinic visit	X	X		X		X				X		
Final Visit												
Informed consent	X											
Review IE Criteria	X	X										
Medical History	X											
Physical Exam	X											
Demographics (age, gender, ethnicity and	X											
Vital Signs (Temp, BP, pulse, RR)	X	X							X			
Height, weight and BMI	X											
Prior & Con Meds	X	X							X			
AEs		X		X		X				X		
Randomization		X										
Vancomycin 250 mg + metronidazole 500 mg (Q8hrs +/- 3 hours)		X	X	X	X	X						
B. Infantis (QD)		X	X	X	X	X	X	X	X	X	X	X
Assess IP and stool compliance		X	X	X	X	X						
Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire		X	X	X	X	X	X	X	X	X	X	X
Patient phone calls		X	X	X	X	X	X	X	X	X	X	X

Urine drug screen test	X	X										
Urine pregnancy test (females of child-bearing potential)	X	X										
Frozen urine aliquots (central lab – Indoxyl Sulfate)		X		X		X						
Hematology and chemistry testing	X	X		X		X				X		
Serum aliquots	X	X		X		X				X		
C-diff and VRE stool screening test	X											
Stool aliquots (no preservatives)	X	X		X		X				X		
Stool aliquots (with ethanol)					X			X				X
Cheek swab for secretor status		X										

Dosing Group 3 Schedule of Events Days 15 - 35

Events	**Washout Period**											
	D15	D16	D17	D18	D19	D20	D21 (+ 2D)	D22	D23	D24	D25	D26
Clinic visit												
Final Visit												
Physical Exam												
Vital Signs (Temp, BP, pulse, RR)												
Prior & Con Meds												
AEs												
Assess IP and stool compliance												
Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire	X	X	X	X	X	X	X	X	X	X	X	X
Patient phone calls												
Frozen urine aliquots (central lab – Indoxyl Sulfate)												

Hematology and chemistry testing												
Serum aliquots												
Stool aliquots (no preservatives)												
Stool aliquots (with ethanol)			X				X					

Dosing Group 4 Schedule of Events Day 1 – 14

Events	**Screening**	**Dosing Days**										
	D0 (≤ 90 days prior to D1)	D1	D2	D3	D4	D5	D6	D7 (+1D)	D8	D9 (+ 1D)	D10	D11
Clinic visit	X	X		X		X				X		
Final Visit												
Informed consent	X											
Review IE Criteria	X	X										
Medical History	X											
Physical Exam	X											
Demographics (age, gender, ethnicity and	X											
Vital Signs (Temp, BP, pulse, RR)	X	X							X			
Height, weight and BMI	X											
Prior & Con Meds	X	X							X			
AEs		X		X		X				X		
Randomization		X										
Vancomycin 250 mg + metronidazole 500 mg (Q8hrs +/- 3 hours)		X	X	X	X	X						
B. Infantis (QD)		X	X	X	X	X	X	X	X	X	X	X
HMO PBCLN-010 (BID)		X	X	X	X	X	X	X	X	X	X	X
HMO (2'FL + LNnT) (Q12 Hours)											X	X
Assess IP and stool compliance		X	X	X	X	X						

Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire		X	X	X	X	X	X	X	X	X	X	X
Patient phone calls		X	X	X	X	X	X	X	X	X	X	X
Urine drug screen test	X	X										
Urine pregnancy test (females of child-bearing potential)	X	X										
Frozen urine aliquots (central lab – Indoxyl Sulfate)		X		X		X						
Hematology and chemistry testing	X	X		X		X				X		
Serum aliquots	X	X		X		X				X		
Stool aliquots (no preservatives)	X	X		X		X				X		
Stool aliquots (with ethanol)					X			X				X
Cheek swab for secretor status		X										

Dosing Group 4 Schedule of Events Days 15-35

Events	**Dosing Days**											
	D15	D16	D17	D18	D19	D20	D21 (+ 2D)	D22	D23	D24	D25	D26
Clinic visit												
Final Visit												
Physical Exam												
Vital Signs (Temp, BP, pulse, RR)												
Prior & Con Meds												
AEs												
HMO (2'FL + LNnT) (Q12 hours)	X	X	X	X	X	X	X	X	X	X	X	X
Assess IP and stool compliance												
Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire	X	X	X	X	X	X	X	X	X	X	X	X
Patient phone calls	X	X	X	X	X	X	X	X	X	X	X	X

Frozen urine aliquots (central lab – Indoxyl Sulfate)												
Hematology and chemistry testing												
Serum aliquots												
Stool aliquots (no preservatives)												
Stool aliquots (with ethanol)			X				X					

Dosing Group 5 Schedule of Events Days 1 – 14

Events	**Screening**	**Dosing Days**										
	D0 (≤ 90 days prior to D1)	D1	D2	D3	D4	D5	D6	D7 (+1D)	D8	D9 (+1D)	D10	D1 (+2D
Clinic visit	X	X		X		X				X		
Final Visit												
Informed consent	X											
Review IE Criteria	X	X										
Medical History	X											
Physical Exam	X											
Demographics (age, gender, ethnicity and	X											
Vital Signs (Temp, BP, pulse, RR)	X	X							X			
Height, weight and BMI	X											
Prior & Con Meds	X	X							X			
AEs		X		X		X				X		
Randomization		X										
Vancomycin 250 mg + metronidazole 500 mg (Q8hrs +/- 3 hours)		X	X	X	X	X						
B. Infantis (QD)		X	X	X	X	X	X	X	X	X	X	X
HMO (2'FL + LNnT)		X	X	X	X	X	X	X	X	X	X	X

Assess IP and stool compliance		X	X	X	X	X						
Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire		X	X	X	X	X	X	X	X	X	X	X
Patient phone calls		X	X	X	X	X	X	X	X	X	X	X
Urine drug screen test	X	X										
Urine pregnancy test (females of child-bearing potential)	X	X										
Frozen urine aliquots (central lab – Indoxyl Sulfate)		X		X		X						
Hematology and chemistry testing	X	X		X		X				X		
Serum aliquots	X	X		X		X				X		
C-diff and VRE stool screening test	X											
Stool aliquots (no preservatives)	X	X		X		X				X		
Stool aliquots (with ethanol)					X			X				X
Cheek swab for secretor status		X										

Dosing Group 5 Schedule of Events Days 15 – 35

Events	**Dosing Days**											
	D15	D16	D17	D18	D19	D20	D21 (+ 2D)	D22	D23	D24	D25	D26
Clinic visit												
Final Visit												
Physical Exam												
Vital Signs (Temp, BP, pulse, RR)												
Prior & Con Meds												
AEs												
HMO (2'FL + LNnT) (Q12 hours)	X	X	X	X	X	X	X	X	X	X	X	X
Assess IP and stool compliance												

Patient journal (Daily Dosing, Bristol Stool Score, and Flavor questionnaire	X	X	X	X	X	X	X	X	X	X	X	X
Patient phone calls	X	X	X	X	X	X	X	X	X	X	X	X
Frozen urine aliquots (central lab – Indoxyl Sulfate)												
Hematology and chemistry testing												
Serum aliquots												
Stool aliquots (no preservatives)												
Stool aliquots (with ethanol)			X				X					

3.5 SAMPLE SIZE

Five dosing groups of 18 subjects, each will be included in this study for a total sample size of 90. The sample size was not determined statistically, but rather represents a typical number for an exploratory study in healthy volunteers.

3.6 TIMING OF ANALYSES

There will be a single analysis of the data at the conclusion of the enrollment of the subjects and the collection of the study endpoints

3.7 ANALYSIS POPULATIONS

Since this study is a safety study, the only analytical population will be individuals who receive at least one dose of either active drug or placebo.

3.8 MISSING DATA

Investigators should make every effort to ensure that loss to follow-up is kept to a minimum and that all data are collected for all subjects at all time points. However, because this is a Phase 1 safety study, no data imputation methods will be used to account for missing data.

3.9 INTERIM ANALYSES AND DATA MONITORING

There are no interim analyses conducted during this study.

4 SUMMARY OF STUDY DATA

All continuous variables will be summarized using the following descriptive statistics: n (non-missing sample size), mean, standard deviation, median, maximum and minimum. The frequency and percentages (based on the non-missing sample size) of observed levels will be reported for all categorical measures. In general, all data will be listed, sorted by dosing group and subject, and when appropriate by visit within subjects. All summary tables will be structured with a column for each dosing group.

4.1 SUBJECT DISPOSITION

A tabulation of subject disposition will be presented by dosing group and overall, including the number screened, the number dosed at each level, the number for primary analysis, the number that withdrew prior to completing therapy, and reasons for withdrawal.

4.2 PROTOCOL DEVIATIONS

Major protocol deviations will be determined at the end of the study when all data have been entered into the clinical database. Protocol violations will be listed by patient.

4.3 DEMOGRAPHIC AND BASELINE VARIABLES

Patient distribution across demographic and baseline characteristics will be tabulated and presented by dose group and overall.

The following demographic and baseline characteristics will be evaluated:

1) Age (calculated as [date of study entry-date of birth]/365.25)
2) Gender
3) Ethnicity
4) Race
5) Physical exam
    - Height
    - Weight
    - BMI
    - Body systems (Normal/Abnormal – not clinically significant/Abnormal – clinically significant/Not done)

- General Appearance
- HEENT
- Heart and cardiovascular
- Dermatologic
- Gastrointestinal
- Musculoskeletal
- Lymphatic
- Neurological
- Respiratory
- Neurological

6) Vital signs
   - Blood pressure
   - Heart rate
   - Respiratory rate
   - Temperature
7) Baseline medical history – Reported conditions will be MedDRA coded and tabulated by system organ class and preferred term.
8) Baseline laboratory assessments

5 PRIMARY ENDPOINT EVALUATION

Changes in the level of B. infantis from baseline will be evaluated by quantitative PCR using primers for specific for the subspecies. This analysis will include descriptive statistics summarized separately by dose group and overall.

6 SECONDARY ENDPOINT EVALUATION

All statistical analyses will be descriptive for the evaluation of the data between and within dose groups and the placebo group

6.1 DNA EXTRACTION FROM HUMAN STOOL, QUANTIFICATION, AND *B. INFANTIS*-SPECIFIC QPCR

Genomic DNA will be extracted from frozen neat stools or stools preserved in ethanol using ZymoBiomics™ 96 MagBead DNA kits (Zymo Research) following manufacturer's instructions. The concentration of the extracted DNA will be measured on a Nanodrop 8000 (Thermo Scientific). To quantify *B. infantis* in stool samples, qPCR will be performed with the Applied Biosystems™ TaqMan™ Fast Advanced Master Mix (ThermoFisher) using 3 ng gDNA as template and primers specific to a *B. infantis* sialidase gene, as previously described (Lawley, et al) Oligonucleotides are listed in Table 1.

Table 1. Oligonucleotides used in the *B. infantis*-specific qPCR

Oligonucleotide	Source	Sequence
B. infantis-specific sialidase; forward primer	Lawley, *et al*., 2017	ATACAGCAGAACCTTGGCCT
B. infantis-specific sialidase; reverse primer	Lawley, *et al*., 2017	GCGATCACATGGACGAGAAC
B. infantis-specific sialidase; probe	Lawley, *et al*., 2017	/FAM/TTTCACGGA/ZEN/TCAC CGG ACCATACG/3IABkFQ/

The qPCR analysis will be reported as genome copies per nanogram of DNA. Log-transformed values will be used to calculate geometric means and standard deviations for each dose group and timepoint. Additionally, a mixed effect analysis for repeated measures of the log-transformed data will be utilized to evaluate differences among dose groups over time. Sidak's multiple-comparison test will be used to evaluate differences in dose groups at discrete timepoints.

6.2 CHANGES IN STOOL MICROBIOTA

6.2.1 Fecal Microbial Diversity

This will include changes in the diversity, composition, and microbial taxa abundance. Microbial evenness and richness will be evaluated across all dose groups.

6.3 WHOLE METAGENOMIC SEQUENCING AND ANALYSES

DNA extracted from human stool samples from days 1, 5, 9, 14, 28, and 35 as described above will be used to prepare libraries for shotgun metagenomic sequencing, and paired-end sequencing (2 x 150 bp) will be performed on an Illumina NovaSeq instrument to generate a target of ~2 million reads per sample (BoosterShot, Diversigen Inc.).

Sequence analyses will follow an established pipeline (Diversigen Inc.). Briefly, sequences will be aligned to a curated database containing all representative genomes in RefSeq for bacteria with additional manually curated strains. Alignments will be made at 97% identity against all reference genomes. Every input sequence will be compared to every reference sequence in the Diversigen Venti database using fully gapped alignment with BURST. Ties will be broken by minimizing the overall number of unique Operational Taxonomic Units (OTUs). For taxonomy assignment, each input sequence will be assigned the lowest common ancestor that is consistent across at least 80% of all reference sequences tied for best hit. Samples with fewer than 10,000 sequences will be discarded. OTUs accounting for less than one millionth of all strain-level markers and those with less than 0.01% of their unique genome regions covered (and < 0.1% of the whole genome) at the species level will be discarded. The counts for each OTU in this filtered table will be normalized to the OTU's genome length, and filtered tables for the normalized counts and relative abundance of each OTU will be generated. Strain-level tracking, including for *B. infantis*, will use the "capitalist" algorithm in BURST to assign reads to genomes in the Diversigen Venti database. Rather than applying a lowest common ancestor approach, the "capitalist" algorithm returns a minimal set of genomes that can explain all the reads in a sample. From that output, an OTU table will be calculated with the read counts per genome per sample. Alpha diversity metrics (observed reads and Shannon diversity) will be calculated using the filtered OTU table rarefied to a read depth of 76,000 using the R package *phyloseq* (v.1.41.0). Bray-Curtis dissimilarity will be calculated on the same filtered data, aggregated at the genus taxonomic level and rarefied to 76,000 reads using the R package *phyloseq* (v1.41.0). The filtered OTU table aggregated at the Family taxonomic level will be used to calculate the top Families among dose groups. All taxa >1% abundance in any dose group will be kept and all taxa <1% among taxa will be grouped into an "Other" category.

Statistical analyses of the data described above are as follows:

Alpha Diversity: the Shannon Diversity Index will be calculated as described above and a repeated measures ANOVA will be used to evaluate differences in dose groups over time. The Sidak post-test will be used to adjust for multiple comparisons. Descriptive statistics including n,

mean/median, standard deviation/interquartile range and 95% confidence intervals will be displayed.

Beta Diversity: The Bray-Curtis Dissimilarity will be evaluated using PERMANOVA , a permutational multivariate analysis of variance. Results will be displayed graphically with a table of p-values for differences between two timepoints.

Changes in microbial taxa abundance: General compositional changes will be reported as geometric mean and geometric standard deviation of taxa >1% relative abundance per dose group identified at the family taxonomic level. Results will be displayed graphically as a composition "stacked bar chart" per dose group.

6.4 CHANGES IN VIABILITY OF PROTEOBACTERIA AND ENTEROCOCCUS

Viability will be determined by the number of colony forming units (CFU) per gram of stool. These data are also compared and reported logarithmically. The data are analyzed using analysis of variance or repeated measures ANOVA with multiple comparisons.

6.5 CHANGES IN THE CARRIAGE LOAD OF ANTIBIOTIC-RESISTANT GENES

Whole metagenomic sequencing (as described above) will allow characterization of functional genes found in the bacterial taxon identified in each dose group. We will identify antibiotic resistance genes by aligning metagenomic sequences against a database that includes known antibiotic resistance genes (for example CARD) and count the presence of these genes in each dose group and compare the instances between dose groups using a chi squared test or exact procedures as needed.

6.6 CHANGES IN IMMUNE MARKERS AS MEASURED IN CYTOKINE AND GROWTH FACTOR LEVEL

Descriptive statistics (mean ± SD, median ± interquartile range) will be calculated by each treatment group and timepoint. Comparisons across time and between dose groups will be made for individuals and groups. Results will be displayed graphically by dose group over time. Data will be analysed on study days Screening, Day 1, Day 3, Day 5, Day 9, Day 14, Day 28 and Day 35.

6.7 SECRETOR STATUS

Secretor status will be determined by analysing DNA collected via a cheek swab. Positive and negative findings will be tabulated by dose group. Positive proportions will be compared among dose groups using a chi-square test for homogeneity.

7 SAFETY EVALUATION

The safety analyses of exposure, AEs and laboratory parameters will include descriptive statistics and will be summarized separately by dose group and overall. Summaries of AEs will be generated by type (AE or SAE), body system and preferred term, severity, and relationship to study product.

7.1 EXPOSURE

Exposure to the test product by visit will be presented in a listing. Subjects will be determined to be "compliant" if all doses are taken; non-compliant if any doses are missed. Descriptive statistics of compliance will be tabulated.

7.2 ADVERSE EVENTS

All reported AEs, will be listed, documenting the course, outcome, severity, and causality to study drug. Verbatim terms on CRFs will be mapped to preferred terms and related system organ class using the Medical Dictionary for Regulatory Activities (MedDRA).

Incidence rates of AEs and the proportion of subjects prematurely withdrawn from the study due to AEs will be shown for all dose groups. Incidence rates will also be displayed based on severity and relationship to study drug. AEs with a relationship of "possibly" or "probably" related will be considered by the Sponsor as "related" to the study drug. Events assessed as "unrelated", "unlikely" related, or where the relationship was not reported will be considered by the Sponsor as "not related" to the study drug. The incidence of SAEs will be provided for each phase. All incidence rates will be categorized and displayed by system organ class and preferred term.

7.3 CLINICAL LABORATORY EVALUATIONS

The laboratory evaluations listed below will be evaluated for clinical lab and medical safety at the following visits: Screening, Day 1, Day 3, Day 5, Day 9, Day 14, Day 28 and Day 35.

- CBC with differential and platelets
- Alkaline phosphatase, alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), total and conjugated bilirubin, albumin and total protein for liver function
- Electrolytes (Na, K, Cl, and HCO_3)
- Glucose, total calcium, magnesium, phosphate, creatinine, and blood urea nitrogen (BUN) for renal function.

All laboratory values will be summarized by descriptive statistics for each dose group and visit; mean ± SD, median ± interquartile range along with counts and percentages for categorical data. Additionally, a table of abnormal laboratory values by subject will be presented.

7.4 VITAL SIGNS

Vital signs (heart rate, respiratory rate, temperature, and blood pressure) are collected at the following visits: Screening, Day 1, Day 3, Day 5, Day 9, Day 14, Day 28, and Day 35. Descriptive statistical summaries will be presented by dose group and visit.

8 REPORTING CONVENTIONS

All data will be reported to the number of significant figures at which they were collected. Summary/descriptive statistics will follow the same convention. P-values and test statistics will be reported to no more than three decimal places.

9 TECHNICAL DETAILS

All analyses will be conducted using SAS Version 9.4, R version 4.2.0, and python version 3.9.16. MedDRA Version 26.0 will be used to code adverse events and medical history conditions.

10 CHANGES IN CONDUCT OF STUDY OR TO PLANNED ANALYSES FROM PROTOCOL

Deviations from the statistical analyses outlined in this plan will be indicated; any further modifications would be noted in the final statistical analyses.

11 REFERENCES

16-CLN-003. (2016). Phase I Safety Study of Human Milk Oligosaccharide Concentrate PBCLN-003 in Healthy Adult Volunteers.

20-CT-001. (2020). 20-CT-001 Study Protocol "Dietary Study of Human Milk Oligosaccharides Concentrate PBCLN-005 with and without Bifidobacterium infantis in Healthy Adult Volunteers".

Ayechu-Muruzabal, V. (2018). Diversity of Human Milk Oligosaccharides and Effects on Early Life Immune Development. Frontiers in Pediatrics, https://doi.org/10.3389/fpred.2018.00239.

Barile, D. (2008). Oligosaccharide prebiotics present in a breast milk based human milk fortifier. Hot Topics in Neonatology. Washington DC .

D. Barile, B. G. (2009). Potential Novel Source for Human Oligosaccharides . American Academy of Pediatrics National Conference and Exhibit. Washington DC .

Duar, R. a. (2020). Choosing a Probiotic for Infant Use: Why Bacterial Strain Matters. Neonatal Intensive Care, 24-26.

Frese, S. (2017). Persistence of Supplemented Bifidobacterium longum subsp. infantis EVC001 in Breastfed Infants. mSphere, e00501-17.

Kelly, R. (1995). Sequence and expression of a candidate for the human Secretor blood group alpha (1,2) fucosyltransferase gene (FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. Journal of Biological Chemistry, 4640-4649.

Lawley, B., Munro, K., Hughes, A., Hodgkinson, A.J., Prosser, C.G., Lowry, D., Zhou, S.J., 1643 Makrides, M., Gibson, R.A., Lay, C., et al. (2017). Differentiation of *Bifidobacterium longum* 1644 subspecies *longum* and *infantis* by quantitative PCR using functional gene targets. PeerJ *5*, 1645 e3375. 10.7717/peerj.3375.

M Ninonuevo, Y. P. (2006). A strategy to annotate the human milk glycome. Journal of Agriculture and Food Chemistry, 7471-7480.

Ramirez, J. (2020). Antibiotics as Major Disruptors of Gut Microbiota. Fronteirs in Cellular and Infectious Microbiology, https://doi.org/10.3389/fcimb.2020.572912.

Smilowtiz, J. L. (2014). Breast Milk Oligosaccharides: Structure-Function Relationships in the Neonate. Annual Reviews in Nutrition, 143-169.

Walsh, C. (2020). From lab bench to formulated ingredient: Characterization, production and commercialization of human milk oligosaccharides. Journal of Functional Foods, 104052