CLINICAL PROTOCOL COVER PAGE

Protocol Title: A randomized, double-blind, placebo-controlled study to investigate the effects of a *Bifidobacterium breve* strain on fat loss in healthy adults

Protocol Date: June 26, 2023

Version: 5

Study Design: Randomized, double-blind, placebo-controlled, 2-arm parallel study

Sponsor:

Sponsor Contact:

CRO:

Medical Director:

PROTOCOL SIGNATURE SHEET

The sponsor and the investigator agree to conduct the study in compliance with the clinical study protocol (and amendments), International Conference on Harmonization (ICH) guidelines for current Good Clinical Practice (cGCP) and applicable regulatory requirements.

Name	**Signature**	**Date**
Sponsor:		
Contract Research Organization:		

1 PROTOCOL SYNOPSIS

Population: Healthy adults

Total number of participants: 100

1.1 Inclusion Criteria

1. Male or female between 20 and 65 years of age, inclusive
2. BMI from 25.0 to 29.9 kg/m^2, inclusive
3. Female participants are not of child-bearing potential, defined as females who have undergone a sterilization procedure (e.g. hysterectomy, bilateral oophorectomy, bilateral tubal ligation, complete endometrial ablation) or have been post-menopausal (natural or surgically) for at least 1 year prior to screening

   Or,

   Females of child-bearing potential must have a negative baseline urine pregnancy test and agree to use a medically approved method of birth control for the duration of the study. All hormonal birth control must have been in use for a minimum of three months. Acceptable methods of birth control include:
   - Hormonal contraceptives including oral contraceptives, hormone birth control patch (Ortho Evra), vaginal contraceptive ring (NuvaRing), injectable contraceptives (Depo-Provera, Lunelle), or hormone implant (Norplant System)
   - Double-barrier method
   - Intrauterine devices
   - Non-heterosexual lifestyle or agrees to use contraception if planning on changing to heterosexual partner(s)
   - Vasectomy of partner at least 6 months prior to screening
4. Self-reported stable body weight for the past 3 months defined as not having gained or lost more than 5 kg of body weight throughout the 3 months prior to baseline
5. Participants with the following body fat percentages:
   - Female: ≥ 25% as determined by Bioelectrical Impedance Analysis (BIA) at screening and confirmed ≥ 30% by Dual-Energy X-Ray Absorptiometry (DXA) at baseline
   - Male: ≥ 15% as determined by BIA at screening and confirmed ≥ 20% by DXA at baseline
6. Agrees to follow the diet and exercise guidelines for the duration of the study
7. Willingness to complete questionnaires, records, and diaries associated with the study, to complete all clinic visits, and provide stool samples
8. Provide voluntary, written, informed consent to participate in the study
9. Healthy as determined by medical history, laboratory results and physical exam as assessed by the Qualified Investigator (QI)

1.2 Exclusion Criteria

1. Women who are pregnant, breastfeeding, or planning to become pregnant during the trial

2. Allergy, sensitivity, or intolerance to the investigational product's active or inactive ingredients
3. Clinically significant abnormal laboratory results at screening as assessed by the QI
4. Current or history of any significant gastrointestinal disease requiring medication (e.g. GERD, gastroenteritis)
5. Irregular sleep schedule
6. Chronic diarrhea or constipation
7. Participants with hypertension and are on antihypertensive medication
8. Type I or Type II diabetes
9. Participants with hyperlipidemia and are on medication
10. Self-reported sleep apnea
11. Self-reported current or pre-existing thyroid condition. Treatment on a stable dose of medication for at least 3 months will be considered by the QI
12. Unstable metabolic disease or chronic diseases as assessed by the QI
13. History of or current diagnosis with kidney and/or liver diseases as assessed by the QI on a case-by-case basis, with the exception of history of kidney stones in participants who are symptom-free for 6 months
14. Significant cardiovascular event in the past 6 months. Participants with no significant cardiovascular event on stable medication may be included after assessment by the QI on a case by case basis
15. Major surgery in the past 3 months or individuals who have planned surgery during the trial period. Participants with minor surgery will be considered on a case-by-case basis by the QI
16. Cancer, except skin cancers completely excised with no chemotherapy or radiation with a follow up that is negative. Volunteers with cancer in full remission for more than five years after diagnosis are acceptable
17. Individuals with an autoimmune disease or are immune-compromised
18. Self-reported HIV-, Hepatitis B- and/or C-positive diagnosis
19. Blood/bleeding disorders as determined by laboratory results
20. Self-reported mental or neuropsychological condition and/or cognitive impairment that, in the QI's opinion, could interfere with study participation
21. Metal implants that may affect the DXA scan results will be assessed on a case-by-case basis by the QI.
22. Current use of prescribed medications listed in Section Prescribed Medications 7.3.1
23. Current use of over-the-counter medications, supplements, foods and/or drinks listed in Section 7.3.2
24. Use of cannabinoid products within 60 days of baseline. History of cannabis used will be assessed on a case by case basis by the QI
25. Use of tobacco products within 60 days of baseline
26. Self-reported alcohol or drug abuse within the last 12 months
27. High alcohol intake (average of > 2 standard drinks per day or > 10 per week)
28. Current employment that calls for shift work or have worked shift work in the last 3 weeks
29. Participation in other clinical research trials 30 days prior to screening
30. Blood donation 30 days prior to screening, during the study, or a planned donation within 30-days of the last study visit
31. Individuals who are unable to give informed consent

32. Any other condition, that, in the opinion of the QI, may adversely affect the participant's ability to complete the study or its measures, or which may pose a significant risk to the participant

1.3 Schedule of Assessments

Procedures/assessments	Visit 1 Screen (Week -6)	Run-in Period Compliance Calls	Visit 2 Week 0	Visit 3 Week 6	Visit 4 Week 12
Informed consent	X				
Review inclusion/exclusion criteria	X		X		
Review medical history	X				
Review daily exercise habits	X				
Review concomitant therapies	X		X	X	X
Biometrics: Height*, weight, waist and hip circumference, heart rate, blood pressure, respiratory rate, and temperature **only be measured at Visit 1*	X		X	X	X
Urine pregnancy test	X		X		
Physical examination			X		
Randomization			X		
CBC, Na, K, Cl, Fe, Mg, Ca, phosphate, HbA1c, fasting glucose, eGFR, creatinine, BUN, AST, ALT, GGT, ALP, TSH* and total bilirubin **only be measured at Visit 1*	X		X		X
Blood Collection: lipid profile and fasting insulin	X		X		X
Urinalysis	X		X		X
DXA			X		X
BIA	X		X		
Stool Collection Kits Dispensed	X			X	
Stool Collection Kit Returned			X		X
Exercise Guideline Review	X	X	X	X	
Daily Bowel Habits Diary Dispensed	X		X	X	
Daily Bowel Habits Diary Returned			X	X	X
3DFR Dispensed	X		X	X	
3DFR Returned			X	X	X
Exercise Diary Dispensed	X		X	X	
Exercise Diary Returned			X	X	X
Subject Diary Dispensed	X		X	X	
Subject Diary Returned			X	X	X
Placebo Dispensed		X			
Placebo Returned			X		
IP Dispensed			X	X	

Procedures/assessments	Visit 1 Screen (Week -6)	Run-in Period Compliance Calls	Visit 2 Week 0	Visit 3 Week 6	Visit 4 Week 12
IP Returned				X	X
Compliance Calculated			X	X	X
Adverse Events		X	X	X	X

2 LIST OF ABBREVIATIONS

3DFR	Three-Day Food Record
AE	Adverse Event
ALP	Alkaline phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BIA	Bioelectrical impedance analysis
BMI	Body Mass Index
BP	blood pressure
BUN	Blood urea nitrogen
Ca	Calcium
CBC	Complete Blood Count
CRF	Case Report Form
Cl	Chloride
DXA	Dual-Energy X-Ray Absorptiometry
EDTA	Ethylenediaminetetraacetic Acid
eGFR	Glomerular Filtration Rate
etc.	*"and so forth"*
e.g.	*"for example"*
et al	*"and others"*
EOS	End of the Study
F/B	Firmicutes/Bacteroidetes
Fe	Iron
g	Gram
GCP	Good Clinical Practice
GGT	Gamma-glutamyl transferase
HbA1c	hemoglobin A1c
HDL-C	High-density lipoprotein cholesterol
HR	Heart Rate
ICF	Informed Consent Form
ICH	International Conference of Harmonization
IP	Investigational product
ITT	Intent to Treat
IRB	Institutional Review Board
K	Potassium
kg	Kilogram
LDL-C	Low-density lipoprotein cholesterol
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MPV	Mean Platelet Volume
Mg	Magnesium
Na	Sodium
NEFA	non-esterified fatty acids
NSAIDs	non-steroidal anti-inflammatory drugs
OTC	over-the-counter

PP	Per protocol
PPIs	Proton Pump Inhibitors
QI	Qualified Investigator
RBC	Red Blood Cells
RDW	Red Blood Cell Distribution Width
SAE	Serious Adverse Event
SOP	Standard operating procedure
SST	Serum Separating Tube
SSRI	Selective serotonin reuptake inhibitors
TG	Triglyceride
TPD	Therapeutics Products Directorate
TSH	Thyroid stimulating hormone
ULN	Upper limit of normal
VLDL	very-low-density lipoprotein
WBC	White Blood Cell
WHO	World Health Organization

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4 INTRODUCTION

In 2016, 1.9 billion adults or 39% of the world's population were classified as overweight (body mass index (BMI) of 25.0 to 29.9 kg/m^2) by the World Health Organization (WHO) (1). Overweight has become a critical issue in North America, with the prevalence of obesity highest in middle-aged adults (2). In the United States (US), the prevalence of overweight and obesity were estimated at 71.6% of the total population in 2015-2016 (3). Consequently, weight loss market value has been increasing, with the US weight loss market worth approximately $72 billion (4). This value is expected to rise as the population becomes more health-conscious and aware of the risks associated with excess body weight.

In early adulthood, excess body weight is a risk factor associated with several health complications later on in life, including type II diabetes, cardiovascular disease, and non-alcoholic fatty liver disease (5, 6). Excess weight gain influences several pathways in the body, altering circulating lipids, glucose, insulin, hemoglobin A1c (HbA1c) levels, and liver function. Increases in visceral adipose tissue lead to a rise in the production of non-esterified fatty acids (NEFA), which decreases insulin sensitivity and play a role in the development of insulin resistance (6). As a result, glucose uptake by muscle tissue is decreased while free fatty acid and glucose production by adipose tissue and liver are increased, respectively (7). These altered pathways increase very-low-density lipoprotein (VLDL) and triglyceride (TG) production in the liver; inducing a decrease in high-density lipoprotein cholesterol (HDL-C) and increase in low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B (8). Additionally, insulin resistance indirectly contributes to the VLDL concentration by lowering lipoprotein lipase activity (7).

Probiotics have been used for decades for maintaining intestinal health, and in recent years probiotics have been proposed for weight management. Previous studies reported a correlation between weight and gut *Bifidobacterium* abundance, showing that the level of *bifidobacterium* in the gut microbiota was lower in obese people (9). *Bifidobacterium breve* (*B. breve*) is a probiotic strain known for its beneficial functions in weight management (10, 11). It has been shown to improve intestinal barrier function, upregulate fat metabolism, and mitigate systemic inflammation (10, 12, 13).

Several studies have also demonstrated that *B. breve* may improve parameters associated with obesity. Supplementation with *B. breve* in obese mice was shown to reduce visceral fat gain and to lower serum cholesterol, glucose, and insulin compared to the control group (14). In a randomized, double-blind, placebo-controlled trial, healthy adults consuming a high-fat diet supplemented with a probiotic mix containing *B. breve* had reductions in body weight and fat mass after 4 weeks of supplementation (15). In another randomized placebo-controlled trial overweight adults were supplemented with *B. breve* for 12 weeks. Results from the study showed significantly lower fat mass along with improvements in liver function and inflammatory markers such as γ-glutamyl transpeptidase (GGT) and high-sensitivity C-reactive protein (CRP) compared to the placebo group (10, 11).

Despite the promising results of probiotics and reductions in body weight and fat mass, weight management is multifactorial and sustained weight loss often has a low success rate. In conjunction with dietary intervention, regular exercise has been proposed to improve body composition, intestinal microbiota diversity, and digestion (16, 17). However, prolonged exercise may disrupt the gastrointestinal tract and reduce the abundance of beneficial bacteria (17). To counteract this effect, the use of probiotics combined with exercise intervention has been proposed for effectively managing weight loss. The combination of aerobic exercise and administration of probiotics has been examined in several clinical trials and it was found that the combination was effective in improving body composition and inflammatory responses (18-20).

The gut microbiota composition influences the effects of foods and drugs. It is reported that individuals who improved their glucose tolerance by taking barley kernel-based bread (responders) had a higher Prevotella/Bacteroides ratio as compared with individuals who did not (non-responders) (21). Several clinical studies indicate that the gut microbiota affects the therapeutic efficacy of checkpoint blockade immunotherapy (22). It is assumed that changes in microbial composition are associated with the anti-obesity effects of *B. breve*.

This study is a randomized, placebo-controlled, clinical trial investigating the effect of *Bifidobacterium breve* supplementation with exercise intervention on fat loss. Participants will be healthy adults between the ages of 20 and 65 with a Body Mass Index (BMI) from 25 to 29.9 kg/m^2.

5 STUDY OBJECTIVES

The objective of this study is to investigate the effect of *Bifidobacterium breve* on fat loss in healthy adults.

Primary outcome:

The difference in change in fat loss from baseline (% or g), as assessed by Dual-Energy X-Ray Absorptiometry (DXA), between *B. breve* and placebo after 12 weeks of supplementation.

Secondary outcomes:

The difference in change from baseline between *B. breve* and placebo in:

1. Body composition (weight, BMI, android/gynoid fat ratio, and muscle mass (% or g) as assessed by DXA after 12 weeks of supplementation
2. Waist circumference, hip circumference, and waist/hip circumference ratio after 6 and 12 weeks of supplementation
3. Lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides) after 12 weeks of supplementation
4. Biomarkers of glycemic control (fasting blood glucose, HbA1c, fasting insulin levels) after 12 weeks of supplementation
5. Liver markers (aspartate transaminase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP)) after 12 weeks of supplementation
6. Frequency of bowel movements after 6 and 12 weeks of supplementation
7. Body composition (weight, BMI, total body fat (% or g), android fat (% or g), gynoid fat (% or g), android/gynoid fat ratio, and muscle mass (% or g) and waist circumference, hip circumference, and waist/hip circumference ratio) in participant groups classified by microbiota composition at week 0
8. Microbiota composition analysis

Safety outcomes:

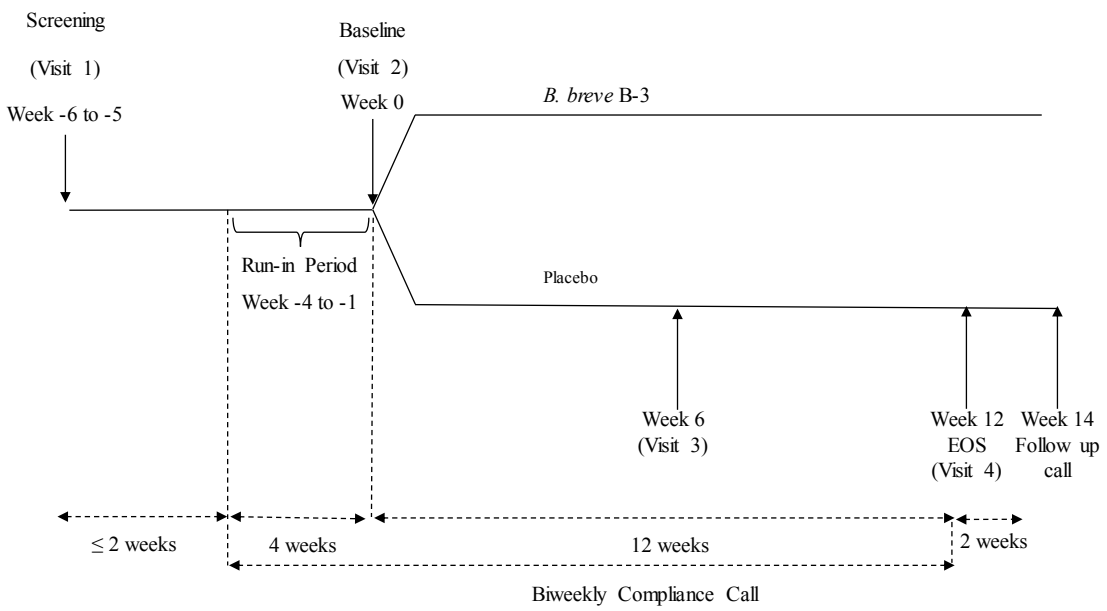
1. Incidence of pre-emergent and post-emergent adverse events

2. Vital signs (blood pressure (BP) and heart rate (HR)), respiratory rate, and temperature
3. Clinical chemistry (total bilirubin, creatinine, blood urea nitrogen (BUN), Na, K, Cl, Ca, Fe, Mg, phosphate, and estimated glomerular filtration rate (eGFR))
4. Hematology (white blood cell (WBC) count with differential (neutrophils, lymphocytes, monocytes, eosinophils, basophils), red blood cell (RBC) count, hemoglobin, hematocrit, platelet count, RBC indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), and mean platelet volume (MPV))
5. Urinalysis (microscopy, glucose, ketones, specific gravity, blood, protein, nitrite, leukocytes, colour, appearance urobilinogen/urine bilirubin, urine pH)

Exploratory analyses

1. Total body fat (%) change during the run-in period as assessed by bioelectrical impedance analysis (BIA)
2. Comparison of total body fat (%) as assessed by BIA and DXA at baseline

6 STUDY DESIGN



This study will be conducted at the KGK Science clinic in London, ON.

The planned sample size for this study is 100 healthy adults. To evaluate primary, secondary, and safety outcomes, study assessments will be conducted as per the Schedule of Assessments in Section 1.3.

Study Arm	Number of Participants
B. breve	N = 50
Placebo	N = 50
Total	N = 100

7 SELECTION OF STUDY POPULATION

This study will enroll 100 healthy male and/or female adults. Each participant must fulfill the inclusion criteria and not meet any of the exclusion criteria as described in Sections 7.1 and 7.2, respectively.

7.1 Inclusion Criteria

1. Male or female between 20 and 65 years of age, inclusive
2. BMI from 25.0 to 29.9 kg/m^2, inclusive
3. Female participants are not of child-bearing potential, defined as females who have undergone a sterilization procedure (e.g. hysterectomy, bilateral oophorectomy, bilateral tubal ligation, complete endometrial ablation) or have been post-menopausal (natural or surgically) for at least 1 year prior to screening

   Or,

Females of child-bearing potential must have a negative baseline urine pregnancy test and agree to use a medically approved method of birth control for the duration of the study. All hormonal birth control must have been in use for a minimum of three months. Acceptable methods of birth control include:

- Hormonal contraceptives including oral contraceptives, hormone birth control patch (Ortho Evra), vaginal contraceptive ring (NuvaRing), injectable contraceptives (Depo-Provera, Lunelle), or hormone implant (Norplant System)
- Double-barrier method
- Intrauterine devices
- Non-heterosexual lifestyle or agrees to use contraception if planning on changing to heterosexual partner(s)
- Vasectomy of partner at least 6 months prior to screening

4. Self-reported stable body weight for the past 3 months defined as not having gained or lost more than 5 kg of body weight throughout the 3 months prior to baseline
5. Participants with the following body fat percentages:
   - Female: ≥ 25% as determined by Bioelectrical Impedance Analysis (BIA) at screening and confirmed ≥ 30% by Dual-Energy X-Ray Absorptiometry (DXA) at baseline
   - Male: ≥ 15% as determined by BIA at screening and confirmed ≥ 20% by DXA at baseline
6. Agrees to follow the diet and exercise guidelines for the duration of the study
7. Willingness to complete questionnaires, records, and diaries associated with the study, to complete all clinic visits, and provide stool samples
8. Provide voluntary, written, informed consent to participate in the study
9. Healthy as determined by medical history, laboratory results and physical exam as assessed by the Qualified Investigator (QI)

7.2 Exclusion Criteria

1. Women who are pregnant, breastfeeding or planning to become pregnant during the trial
2. Allergy, sensitivity, or intolerance to the investigational product's active or inactive ingredients
3. Clinically significant abnormal laboratory results at screening as assessed by the QI
4. Current or history of any significant gastrointestinal disease requiring medication (e.g. GERD, gastroenteritis)
5. Irregular sleep schedule
6. Chronic diarrhea or constipation
7. Participants with hypertension and are on antihypertensive medication
8. Type I or Type II diabetes
9. Participants with hyperlipidemia and are on medication
10. Self-reported sleep apnea
11. Self-reported current or pre-existing thyroid condition. Treatment on a stable dose of medication for at least 3 months will be considered by the QI
12. Unstable metabolic disease or chronic diseases as assessed by the QI
13. History of or current diagnosis with kidney and/or liver diseases as assessed by the QI on a case-by-case basis, with the exception of history of kidney stones in participants who are symptom-free for 6 months

14. Significant cardiovascular event in the past 6 months. Participants with no significant cardiovascular event on stable medication may be included after assessment by the QI on a case by case basis
15. Major surgery in the past 3 months or individuals who have planned surgery during the trial period. Participants with minor surgery will be considered on a case-by-case basis by the QI
16. Cancer, except skin cancers completely excised with no chemotherapy or radiation with a follow up that is negative. Volunteers with cancer in full remission for more than five years after diagnosis are acceptable
17. Individuals with an autoimmune disease or are immune-compromised
18. Self-reported HIV-, Hepatitis B- and/or C-positive diagnosis
19. Blood/bleeding disorders as determined by laboratory results
20. Self-reported mental or neuropsychological condition and/or cognitive impairment that, in the QI's opinion, could interfere with study participation
21. Metal implants that may affect the DXA scan results will be assessed on a case-by-case basis by the QI.
22. Current use of prescribed medications listed in Section Prescribed Medications 7.3.1
23. Current use of over-the-counter medications, supplements, foods and/or drinks listed in Section 7.3.2
24. Use of cannabinoid products within 60 days of baseline. History of cannabis used will be assessed on a case by case basis by the QI
25. Use of tobacco products within 60 days of baseline
26. Self-reported alcohol or drug abuse within the last 12 months
27. High alcohol intake (average of > 2 standard drinks per day or > 10 per week)
28. Current employment that calls for shift work or have worked shift work in the last 3 weeks
29. Participation in other clinical research trials 30 days prior to screening
30. Blood donation 30 days prior to screening, during the study, or a planned donation within 30-days of the last study visit
31. Individuals who are unable to give informed consent
32. Any other condition, that, in the opinion of the QI, may adversely affect the participant's ability to complete the study or its measures, or which may pose a significant risk to the participant

7.3 Concomitant Medications

Participants who are taking any prescribed medications that are considered not to affect the study outcomes must agree to maintain their current dosing regimen during the study unless otherwise recommended by their family physician.

7.3.1 Prescribed Medications

Participants on the following prescribed medications and/or treatments will be excluded during enrollment unless they have been taken off these therapies by their family physician. In the latter event, the frequency of use and/or dosage may be considered by the QI on a case by case basis prior to recommending an appropriate washout or their enrollment in the study.

1. Beta-blockers and thiazide diuretics (within 4 weeks of baseline)
2. Weight loss medication (within 4 weeks of baseline)
3. Lipid-lowering medications (within 4 weeks of baseline)
4. Anticoagulants and coagulants (within 4 weeks of baseline)
5. Sleep medication
6. Selective serotonin reuptake inhibitors (SSRI)
7. Antibiotics
8. Non-steroidal anti-inflammatory drugs (NSAIDs)
9. Proton pump inhibitors (PPIs)
10. Metformin (unless on a stable dose for the last 6 months)

7.3.2 Over-the-counter Medications, Supplements and Foods/Drinks

Participants who are currently consuming the following over-the-counter (OTC) medications, supplement, and food/drinks will not be allowed to participate unless willing to undergo the specified washout period prior to their baseline visit and agree not to take the supplements during the study. Other OTCs supplement and food/drinks will require a case-by-case assessment by the QI based on dose and/or frequency of use to determine adequate washout.

1. Probiotics, prebiotics, and synbiotics (30 days prior to baseline and throughout the study) (see appendix 17.1 for full lists)
2. OTC NSAIDs (PRN use is acceptable)
3. OTC blood pressure medication or supplements (within 4 weeks of baseline)
4. Lipid metabolising supplements (within 4 weeks of baseline)
    a. Fish oil and omega-3 supplements
    b. Red yeast rice
    c. Plant sterols and stanols
5. OTC medication or supplements marketed for weight loss (within 4 weeks of baseline)
6. Vitamin E supplements (within 4 weeks of baseline)
7. Coagulant/anticoagulant supplements (within 4 weeks of baseline)
8. PPIs

7.4 Washout Periods

Please refer to section 7.3.2 for washout periods for OTC medications, suppliments and drinks.

7.5 Early Withdrawal

Personal reasons

As stated in the Informed Consent Form, a participant may withdraw from the study for any reason at any time.

Removal by Qualified Investigator

Participant discontinuation should be considered at the discretion of the qualified investigator. The circumstances of any discontinuation must be documented in detail in the participant file and final report. If possible, the evaluations planned for the end of treatment will be carried out at the time when the participant is withdrawn from the study.

Criteria for removal of participants from the study includes:

Clinical reasons

A participant may be withdrawn from the study if, in the opinion of the qualified investigator, it is not in the participant's best interest to continue. Any participant who experiences a serious adverse event (SAE) may be withdrawn from the trial at the discretion of the qualified investigator. A participant will also be withdrawn due to adverse events causing clinically significant illness or the need for prohibited medication(s) during the trial. Any female participant who becomes pregnant during the course of the trial will be withdrawn and will be followed up until giving birth.

Protocol violation

Any participant found to have entered this study in violation of the protocol will be discontinued from the study at the discretion of the qualified investigator. This will include any participant found to have been inappropriately enrolled (did not meet eligibility criteria). Participant non-compliance includes failure to show up for study visits, failure to take the investigational product as directed, or refusal to undergo study visit procedures. Participants who are found to be taking prohibited medications or supplements without the knowledge of the qualified investigator will also be withdrawn. Any major protocol deviations (i.e. those that increase the risk to participants and/or compromise the integrity of the study or its results) will result in participant discontinuation.

Particiapnt Replacement

For all early withdrawals, a participant leaving the study prematurely or in the event of participant removal will NOT be replaced by another. It is understood by all concerned that an excessive rate of withdrawals can render the study un-interpretable, thus unnecessary withdrawal of participants should be avoided.

8 INVESTIGATIONAL PRODUCT

8.1 Manufacturing and Storage

The investigational product will be provided to KGK by the Sponsor. The investigational product will be carefully stored at the study site in a lockable, limited access area, accessible only to study team personnel in compliance with pertinent regulations. Only authorized persons will have access to the investigational product. The products will be stored at room temperature and will not be exposed to direct sunlight or heat. The investigational products will be kept in a locked investigational product storage room at KGK Science Inc. on receipt. An accountability log will be kept for investigational products.

All unused investigational product will be returned to the study sponsor by KGK (at the sponsor's expense) or destroyed on receipt of written confirmation from the sponsor at study closeout (within one month of last participant visit).

Manufactured by:

Morinaga Nutritional Foods, Inc.

8.2 Labelling and Coding

The investigational product will be labeled according to the requirements of ICH-GCP guidelines and applicable local regulatory guidelines. The investigational product will be randomized and coded by an unblinded person at KGK who is not involved in data collection or analysis.

8.3 Investigational Product (*Bifidobacterium breve*)

Dietary Ingredient	Quantity (Qty)
Bifidobacterium breve	10 billion/capsule

Non-medical ingredients: corn starch, milk protein hydrolysate, hypromellose, sodium alginate, calcium carbonate, and calcium phosphate

Placebo

Ingredients: corn starch, milk protein hydrolysate, hypromellose, sodium alginate, calcium carbonate, and calcium phosphate

8.4 Directions

Participants will be instructed to take 2 capsules of placebo for 4 weeks during the run-in period. On day 1 participants will be instructed to take either 2 capsules of placebo or *B. breve* (20 billion) in the morning after breakfast for 12 weeks. Clinic staff will instruct participants to save all unused and open packages and return them to KGK for a determination of compliance. If a dose is missed participants will be instructed to take their dose when they remember with their next meal. If an entire day is missed, participants are instructed to record it as missed and continue on with the dose as instructed the next day. Participants will be advised not to exceed 2 capsules daily.

8.5 Rescue Medication

Rescue medication is not applicable for this study.

8.6 Randomization

A randomization schedule will be created and provided to the Investigator indicating the order of randomization. Each participant will be assigned a randomization code according to the order of the randomization list generated using www.randomization.com. Enrolled participants will be randomized to the different study arms at Day 0.

8.7 Blinding and Allocation Concealment

Concealment of the allocation of treatment will be employed through the use of opaque sealed envelopes, each labeled with a randomization number. Each envelope will contain information regarding the treatment associated with each randomization number. These envelopes will be readily available for the investigator to open in the event that it becomes necessary to know which product a participant is taking for the sake of the participant's health care.

Unblinding should not occur except in the case of emergency situations. In the event that a serious adverse event occurs, for which the identity of the investigational product administered is necessary to manage the participant’s condition, the treatment received by the participant will be unblinded and the investigational product identified. The sponsor must be notified of any unblinding within 24 hours. Details of participants who are unblinded during the study will be included in the Final Report.

9 STUDY ASSESSMENTS

9.1 Screening (week -6 to -5; Visit 1) *

** At the discretion of the Qualified Investigator, any participants falling outside of the screening window (week -6 to -5) due to scheduling issues will be asked to repeat eligibility/screening procedures prior to randomization at baseline.*

Participants will arrive at the clinic for screening assessments in a 12-hour or greater fasted state.

An informed consent form will be given to the potential volunteer. They will be required to read the information and will be given the opportunity to seek more information if needed, or the option of taking the consent form home to review prior to making their decision. If agreeable, the volunteer will sign the consent form and receive a duplicate of the signed copy. Once consent has been obtained, the screening visit will proceed. Each volunteer will be sequentially assigned a screening number to be entered in the screening and enrollment log.

Screening assessments include:

1. Review medical history, concomitant therapies, and daily exercise habits
2. Assess inclusion and exclusion criteria
3. Urine collection
    a. Urine pregnancy test for female potential participants that are of child-bearing potential
    b. Urinalysis (microscopy, chemical, urobilinogen/urine bilirubin)
4. Seated resting blood pressure, heart rate, respiratory rate, and temperature measurements
5. Weight, height (BMI calculation), waist circumference and hip circumference measurements
6. Body fat composition as determined by BIA
7. Collect fasted blood samples for analysis of CBC, Na, K, Cl, Fe, Mg, Ca, phosphate, HbA1c, fasting glucose, eGFR, creatinine, BUN, AST, ALT, GGT, ALP, TSH, total bilirubin, lipid profile, and fasting insulin
8. Dispense Three-Day Food Record (3DFR)
9. Dispense Daily Bowel Habits Diary
10. Dispense Exercise Diary
11. Dispense Subject Diary
12. Dispense stool collection kits

The next visit will be scheduled for potentially eligible participants for baseline (Week 0).

Reminders for participants prior to their next in-clinic visit:

1. Return to the clinic in a fasted state (12 hours or greater)
2. Abide by instructions for prescribed medications as per Section 7.3.1 and OTC medications, supplements, and food/drinks as per Section 7.3.2
3. Limit alcohol intake (<2 standard drinks per day or <10 per week)
4. Return stool collections
5. Complete and return Daily Bowel Habits Diary
6. Complete and return 3DFR Diary
7. Complete and return Exercise Diary
8. Complete and return Subject Diary

9.2 Run-in period – 4 weeks (Week -4 to Week -1)

Eligible participants will be contacted to undergo a 4 week (28 days) run-in period, during which they will be consuming the placebo and following an exercise intervention. Participants will come to the clinic to pick up the placebo. Compliance calls will be completed throughout the run-in period at fixed intervals.

Instructions during the run-in period:

1. Begin consuming placebo according to dosing instructions provided
2. Begin exercise regimen according to instructions provided (20 min aerobic exercise/day; 3 days/week) and record exercise in an exercise diary
3. Complete Daily Bowel Habits Diary, 3DFR and subject diary
4. Record adverse events

9.3 Compliance Call

Participants will receive bi-weekly calls throughout the run-in and study period to confirm compliance on exercise intervention, concomitant therapies, and IP consumption. Participants will also be reminded to bring in their stool samples at baseline visit.

9.4 Baseline (Visit 2, Week 0)

Eligible participants (compliant with the exercise intervention and placebo consumption during the run-in period) will return to the clinic fasted for 12 hours with completed subject diaries, food records, daily bowel habit diaries, exercise diaries, and stool collections, for baseline assessments. Compliance calls will be made at fixed intervals (bi-weekly). Participants who have not provided a stool sample within 3 days of visit 2 and participants who have significant weight loss (> 0.7 kg/week) during the run-in period will be withdrawn from the study.

Baseline (Day 0) assessments include:

1. Review concomitant therapies and adverse events
2. Assess inclusion and exclusion criteria
3. Collect remaining placebo and calculate compliance
4. Physical examination
5. Collect and store stool samples
6. Collect and review Daily Bowel Habits Diary
7. Collect and review 3DFR
8. Collect and review Exercise Diary
9. Collect and review Subject Diary
10. Urine collection
    a. Urine pregnancy test for female participants that are of child-bearing potential
    b. Urinalysis (microscopy, chemical, urobilinogen/urine bilirubin)
11. Seated resting blood pressure, heart rate, respiratory rate, and temperature measurements
12. Weight (BMI calculation), waist and hip circumference measurements (waist/hip circumference ratio)

13. Randomization of eligible participants
14. Body fat composition as determined by BIA
15. DXA scan
16. Collect fasted blood samples for analysis of CBC, Na, K, Cl, Fe, Mg, Ca, phosphate, HbA1c, fasting glucose, eGFR, creatinine, BUN, AST, ALT, GGT, ALP, total bilirubin, lipid profile, and fasting insulin
17. Dispense investigational product and instruct participants on use
18. Dispense Daily Bowel Habits Diary
19. Dispense 3DFR
20. Dispense Exercise Diary
21. Dispense Subject Diary

The next visit will be scheduled for week 6.

Reminders for participants prior to their next in-clinic visit:

1. Return to the clinic in a fasted state (12 hours or greater)
2. Maintain exercise intervention (20 min aerobic exercise x 3 days/week)
3. Abide by instructions for prescribed medications as per Section 7.3.1 and OTC medications, supplements, and food/drinks as per Section 7.3.2
4. Limit alcohol intake (<2 standard drinks per day or <10 per week)
5. Complete and return Daily Bowel Habits Diary
6. Complete and return 3DFR Diary
7. Complete and return Exercise Diary
8. Complete and return Subject Diary
9. Return unused IP

9.5 Visit 3 (Week 6, 42 ± 3 days)

Participants will return to the clinic fasted for 12 hours, with the unused product, in addition to completed subject diaries, food records, daily bowel habits diaries andexercise diaries, for visit 3. Compliance calls will be made at fixed intervals.

Visit 3 assessments include:

1. Review concomitant therapies and adverse events
2. Collect unused investigational product; calculate compliance
3. Collect and review Subject Diary
4. Collect and review 3DFR
5. Collect and review Daily Bowel Habits Diary
6. Collect and review Exercise Diary
7. Seated resting blood pressure, heart rate, respiratory rate, and temperature measurements
8. Weight (BMI calculation), waist and hip circumference measurements (waist/hip circumference ratio)
9. Dispense investigational product and instruct participants on use
10. Dispense Subject Diary
11. Dispense 3DFR
12. Dispense Daily Bowel Habits Diary

13. Dispense Exercise Diary
14. Dispense stool collection kits

The next visit will be scheduled for week 12 (EOS).

Reminders for participants prior to their next in-clinic visit:

1. Return to the clinic in a fasted state (12 hours or greater)
2. Maintain exercise intervention (20 min aerobic exercise x 3 days/week)
3. Abide by instructions for prescribed medications as per Section 7.3.1 and OTC medications, supplements, and food/drinks as per Section 7.3.2
4. Limit alcohol intake (<2 standard drinks per day or <10 per week)
5. Complete and return stool collections
6. Complete and return Daily Bowel Habits Diary
7. Complete and return 3DFR
8. Complete and return Exercise Diary
9. Complete and return Subject Diary
10. Return unused IP

9.6 Visit 4 - End of Study (Week 12, 84 ± 3 days)

Participants will return to the clinic fasted for 12 hours or greater with the unused product, in addition to completed subject diaries, food records, daily bowel habits diaries, exercise diaries, and stool collections, for assessments. Compliance calls will be made at fixed intervals.

Visit 4 assessments include:

1. Review concomitant therapies and adverse events
2. Return the unused investigational product in the original packaging and remnants; calculate compliance
3. Collect and store stool samples
4. Collect and review Subject Diary
5. Collect and review 3DFR
6. Collect and review Daily Bowel Habits Diary
7. Collect and review Exercise Diary
8. Urine collection
    a. Urinalysis (microscopy, chemical, urobilinogen/urine bilirubin)
9. Seated resting blood pressure, heart rate, respiratory rate, and temperature measurements
10. Weight (BMI calculation), waist and hip circumference measurements (waist/hip circumference ratio)
11. Collect fasted blood samples for analysis of CBC, Na, K, Cl, Fe, Mg, Ca, phosphate, HbA1c, fasting glucose, eGFR, creatinine, BUN, AST, ALT, GGT, ALP, total bilirubin, lipid profile, and fasting insulin
12. DXA scan

9.7 Follow-up (Week 14)

Participants will receive a follow-up call at week 14 for adverse event reporting.

10 CLINICAL ASSESSMENTS AND PROCEDURES

Calculations or measurements of specific parameters are required, as indicated in the schedule of assessments in Section 1.3. Instructions for determining these parameters are provided in the following sections.

10.1 Height and Weight

Weight measurements will be performed with shoes removed and bladder empty on calibrated scales at all visits.

At least two separate measurements will be taken at each visit. If the two measurements are more than 0.5 kg (1.1 lbs) apart, a third measurement will be taken. The two closest values will be selected and entered into the database.

Measurement of height will be performed with the participant's shoes removed. The participant's knees will be straightened, and head held upright.

10.2 Circumference Measurements

Waist circumference

The waist circumference of the participant is to be measured when standing at the part of the trunk located midway between the lower costal margin (bottom of lower rib) and the iliac crest (top of pelvic bone). The measurer is to stand beside the participant and fit the tape tightly against the skin without compressing underlying soft tissues. The circumference should be measured at the end of a normal expiration. At least two separate measurements are to be taken at each visit. If the two measurements differ by more than 10%, a third measurement is to be taken. The two closest values will then be entered into the database. All measurements will be recorded to the nearest 0.1 cm.

The height of the waist should be recorded at baseline, and then used to determine the waist throughout the study in order to ensure a consistent measurement at follow-up visits.

Hip circumference

While the participant is standing upright, a measuring tape will be placed around the greater trochanteric prominence (the widest part of the hips). The tape will be held firmly, but not pressing the tape into the skin. When the tape is parallel to the floor, the reading will be recorded. At least two separate measurements

are to be taken at each visit. If the two measurements differ by more than 10% a third measurement will be taken. The two closest values are to be entered into the database.

To ensure consistent measurement at follow-up visits, the height of the identified hips will be recorded at the first visit and then used at subsequent visits to determine the hip circumference throughout the study.

Waist/hip ratio

The waist-to-hip ratio will be assessed by comparing the waist circumference to hip circumference at each study visit.

10.3 Blood Pressure and Heart Rate

In-office, seated, resting blood pressure assessment:

> The participant should be seated comfortably with their back supported and the upper arm bared without restrictive clothing. Feet should be flat on the floor and legs will not be crossed. The participant will rest in this position for at least 5 minutes prior to the first reading.

At screening:

> Seated blood pressure will be checked in both arms and if different the arm with the higher systolic blood pressure reading will be taken for measurements. If the systolic blood pressure is the same in both arms, the arm with the higher diastolic blood pressure will be used. If both are equal, then the left arm will be used. In the chosen arm, a second measurement will be taken at least 1 minute from the first measurement. If a difference of more than 8 mmHg exists between the two readings a third reading will be taken. An average of the two lowest readings from the chosen arm will be taken for the determination of inclusion into the study. As per the QI's opinion, a high office blood pressure should be rechecked manually after the participant is given a glass of water and is seated for 15 min. Also, participant should be queried about their usual blood pressure.
>
> The arm chosen for use at the initial visit will be documented in the study file and used in all subsequent visits.

At study visits:

> Once enrolled in the study, BP will be measured in the chosen arm. Three readings will be made, averaged and recorded in the chart.

Heart Rate (beats/min) will be measured using the reading on the automated blood pressure monitor, or manually by the clinical coordinator placing their index finger on the participant's radial artery while observing a timer and counting the number of beats over 30 seconds and then multiplying the number by two. This is repeated for a total of three measurements.

10.4 Temperature and Respiratory Rate

Oral temperature will be measured after ensuring that the participant did not consume any hot or cold beverages for a minimum of 20 minutes. The temperature will be taken by placing the thermometer with probe cover under the participant's tongue within the sublingual pocket. The participant will be instructed to lower his/her tongue and close their lips during the measurement. The participant will be instructed to not talk during the measurement until the thermometer indicates that the final temperature has been recorded. Once the recording is complete the thermometer will be removed from the participant's mouth and probe cover discarded.

Respiratory rate will be measured and recorded by counting the number of breaths a participant takes in one minute with the participant at rest.

10.5 Blood Sample Collection

An appropriately trained and qualified phlebotomist will perform the venipuncture procedure to collect the necessary blood samples. Participants will be placed in a comfortable seated position with their desired arm, at the phlebotomist's discretion, fully extended and supported with a pillow. A tourniquet will be applied 3-4 inches above the elbow with the participants opening and closing their fist a few times to allow the phlebotomist to manually determine the approximate size, depth, and location of the vein. Following the site of the venipuncture being appropriately sterilized, the phlebotomist will collect the sample using the vacutainer system according to the relevant laboratory order requirement. Once the collection is complete a cotton ball will be immediately placed on the venipuncture site which will be periodically checked to ensure clotting has begun at which point a clean cotton ball will be applied and secured with surgical tape. All collected sample tubes will be labeled with subject identification codes, visit number, project code, DOB (Date of Birth), gender, date and time of draw.

10.6 Stool Sample Collection

Participants will be instructed on stool sample collection at screening. They will be provided with a stool sample collection kit and instructions at screening and at visit 3. All collected samples tubes will be labeled with subject identification codes, visit number, project code, DOB (Date of Birth), gender, date and time of collection.

10.7 Bioelectrical Impedance Analysis (BIA)

The Bioelectrical Impedance Analysis will be conducted at KGK clinic (London, ON) at the screening and baseline visits. The BIA assessment will be carried out and fully explained by trained clinic coordinators upon participants arrival at the clinic. The total body water will be determined from the resistance of the alternate electric current through the body's water pool. The value obtained will be used to estimate the body fat mass percentage.

10.8 Dual-Energy X-Ray Absorptiometry (DXA) Scan

The Dual-Energy X-Ray Absorptiometry Scan will be conducted at KGK clinic (London, ON) at visits 2 (week 0), and 4 (week 12) by trained technicians. Body tissue density will be measured by a form of X-ray radiation and converted into body fat and muscle mass percentage for assessment. Prior to the day of the DXA assessment, the coordinators will remind the participants to limit the amount of water and food consumption accordingly. A full body composition DXA scan will expose the participant to a total dose of <10uSV, depending on the size of the participant. This amount is less than a person is exposed to on an east to west coast flight within the USA and similar to the normal background radiation received over the course of a single day level (23, 24).

10.9 Daily Bowel Habit Diary and Bristol Stool Scale

The participants will be instructed to fill and complete the Daily Bowel Habit Diary for assessment at visits 2 (week 0), 3 (week 6), and 4 (week 12). The diary is a 7-item questionnaire that evaluates each bowel movement experienced. The Bristol Stool Scale is a 7-item image scale that measures the shape and consistency of stool.

10.10 3-Day Food Record (3DFR)

The participants will be instructed to complete an online 3-day food record called Libro, by Nutritics, for assessment on visits 2 (week 0), 3 (week 6), and 4 (week 12). Clinic coordinators will create an online access for each participant void of all personal information to protect any confidential participant information. The recorded food and drink consumption will be used for daily calories, macronutrients, micronutrients, and compliance calculation. Participants will be counselled accordingly after their food record has been review by a trained clinic staff. In the event the access to Libro is disrupted, participants will be instructed to record their food consumption on a paper food record, and once access is restored data will be incorporated into the database.

10.11 Exercise Diary

The participants will be instructed to complete an online weekly exercise diary. Clinic coordinators will create an online access for each participant void of all personal information to protect any confidential participant information. The recorded exercise will be used for compliance calculation. Participants will be counselled accordingly after exercise record has been reviewed by a trained clinic staff. In the event that online access is disrupted, participants will be instructed to record their exercise in a paper diary, and once access is restored data will be incorporated into the database.

10.12 Compliance

Compliance to supplement

Compliance will be assessed by counting the returned unused study product at each visit. Compliance is calculated by determining the number of dosage units taken divided by the number of dosage units expected to have been taken multiplied by 100.

$$\frac{number\ of\ dosage\ units\ taken}{number\ of\ dosage\ units\ expected\ to\ have\ been\ taken} \times 100\%$$

In the event of a discrepancy between the information in the treatment diary and the amount of study product returned, compliance will be based on the product returned unless an explanation for loss of product has been provided. Participants found to have compliance of <80% or >120% will be counseled.

Compliance to exercise

Aerobic exercise 20 minutes per day, 3 days a week
Weekly maximum score = 3 (compliant 3 days/week)

Compliance is calculated weekly by dividing the actual weekly score by the maximum weekly score multiplied by 100.

$$\frac{actual\ score}{3} \times 100\%$$

Participants found to have compliance of <80% will be counseled. Compliance of <70% will be considered as non-compliant and any participant demonstrating non-compliance for two consecutive weeks will be withdrawn from the study.

10.13 Laboratory Analyses

12 hours fasting blood samples will be drawn from the participants at screening (Visit 1), baseline visit (Visit 2), and at the End of Study visit (Visit 4) as indicated in the schedule of assessments in Section 1.3.

Protection of subject confidentiality will extend to all data generated from the assaying of these samples. These samples will be alphanumerically coded and the persons performing the analysis will not be aware of the subject's identity or the product they received.

At screening (Visit 1), 18 mL of 12 hour fasting whole blood will be collected in:

1. Two 4 mL EDTA vacutainer tubes for:
   a. CBC analysis (One tube)
   b. HbA1c analysis (one tube)

2. Two 5 mL SST vacutainer tubes to generate serum for:
   a. Na, K, Cl, Fe, Ca, Mg, phosphate, fasting glucose, TSH, Lipid Panel, creatinine, eGFR, BUN, GGT, ALP, AST, ALT, and total bilirubin analysis (one tube)
   b. Fasting Insulin (One tube)

3. Urine will be collected for urine pregnancy test of women of childbearing potential and urinalysis

At baseline (Visit 2), 18 mL of 12 hours fasting blood will be collected in:

1. Two 4 mL EDTA vacutainer tubes for:
   a. CBC analysis (one tube)
   b. Hb1-Ac analysis (one tube)

2. Two 5 mL SST vacutainer tubes to generate serum for:
   a. Na, K, Cl, Fe, Ca, Mg, phosphate, fasting glucose, Lipid Panel, creatinine, eGFR, BUN, GGT, ALP, AST, ALT, and total bilirubin analysis (one tube)
   b. Fasting Insulin (One tube)

3. Urine will be collected for urine pregnancy test of women of childbearing potential and urinalysis.

4. Fecal Sample also will be collected for analysis

At the end of the study (Visit 4), 18 ml of 12 hours fasting blood will be collected in:

1. Two 4 mL EDTA vacutainer tubes for:
   a. CBC analysis (one tube)
   b. Hb1-Ac analysis (one tube)

2. Two 5 mL SST vacutainer tubes to generate serum for:
   a. Na, K, Cl, Fe, Ca, Mg, phosphate, fasting glucose, Lipid Panel, creatinine, eGFR, BUN, GGT, ALP, AST, ALT, and total bilirubin analysis (one tube)
   b. Fasting Insulin (One tube)

3. Urine will be collected for urinalysis

4. Fecal Sample also will be collected for analysis

The total blood volume collection for the laboratory assessments listed above will be approximately 54 mL, over the period from screening to end of study. At any study visit, blood loss per volunteer is not expected to exceed 18 ml. Additional blood samples may be collected during the study in order to perform or repeat laboratory tests outlined in the Schedule of Assessments if needed.

Dynacare a central laboratory will be used in this study to measure blood parameters and urinalysis. Another lab will be used to process fecal samples.

Urine pregnancy test will be performed at the KGK Science clinic site.

10.14 Termination of the Trial

In the case of premature termination of the trial, participating investigators/participants, and the Institutional Review Board must be promptly informed of the termination. In the event of early termination, as many assessments will be completed as agreed upon by participant

10.15 Protocol Amendments

If amendments to the study protocol are required after approval such changes will be captured in writing the reasons for the change documented and signed and dated by the sponsor. Any such amendments may be subject to IRB and Health Canada review/approval prior to implementation. Exception: if it becomes necessary to alter the protocol to eliminate an immediate hazard to participants, an amendment may be implemented prior to IRB approval. In this circumstance, the Investigator must notify IRB and Health Canada in writing within five (5) working days of the implementation.

11 SAFETY INSTRUCTIONS AND GUIDANCE

11.1 Adverse Events and Laboratory Abnormalities

11.1.1 Adverse Events

An adverse event (AE) is any untoward medical occurrence in a clinical investigation participant who has been administered an investigational product and does not necessarily have a causal relationship with this treatment. An AE can be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a product, whether or not it is considered related to that product. Pre-existing conditions that worsen during a study are to be reported as AEs.

During the study, participants should record any adverse effects in their diary. At each visit the participant will be asked, "Have you experienced any difficulties or problems since I saw you last?". Any adverse events (AEs) will be documented in the study record and will be classified according to the description, duration, intensity, frequency, and outcome. The qualified investigator will assess any AEs and decide causality.

Intensity of AEs will be graded on a three-point scale (mild, moderate, severe) and reported in detail in the study record.

Mild:	Awareness of event but easily tolerated
Moderate:	Discomfort enough to cause some interference with usual activity
Severe:	Inability to carry out usual activity

The causality relationship of investigational product to the adverse event will be assessed by the qualified investigator as either:

Most probable: There is a reasonable relationship between the investigational product and AEs. The event responds to withdrawal of investigational product (dechallenge) and recurs with rechallenge when clinically feasible.

Probable: There is a reasonable relationship between the investigational product and AEs. The event responds to dechallenge when clinically feasible.

Possible: There is a reasonable relationship between the investigational product and AEs. Dechallenge information is lacking or unclear.

Unlikely: There is a temporal relationship to the investigational product administration but there is no reasonable causal relationship between the investigational product and the AEs.

Not related: There is no temporal relationship to investigational product administration or there is a reasonable causal relationship between non-investigational product, concurrent disease or circumstance and the AEs.

11.1.2 Serious Adverse Event

A serious adverse event (SAE) is any experience that suggests a significant hazard, contraindication, side effect or precaution. It is any AE that results in any of the following outcomes:

1. Death
2. A life-threatening adverse event
3. Inpatient hospitalization or prolongation of existing hospitalization
4. A persistent or significant disability or incapacity
5. A congenital anomaly/birth defect in the offspring of a participant who received the study treatment

Important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the participant or may require intervention to prevent one of the outcomes listed above. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or the development of drug dependency or drug abuse.

11.1.3 Unexpected Adverse Reaction

An unexpected adverse reaction is an adverse reaction, the nature and severity of which is not consistent with the applicable product information (e.g., Investigator's Brochure for an unapproved investigational product or package insert/summary of product characteristics for an approved product).

11.1.4 Laboratory Test Abnormalities

The investigator must assess the clinical significance of all abnormal laboratory values as defined by the compendium of normal values for the reference laboratory.

Any treatment emergent abnormal laboratory result which is clinically significant, i.e., meeting one or more of the following conditions, should be recorded as a single diagnosis on the AEs form in the study record:

1. Accompanied by clinical symptoms
2. Leading to interruption or discontinuation of the investigational product
3. Requiring a change in concomitant therapy

This applies to any protocol and non-protocol specified laboratory result from tests performed after the first dose of the investigational product, which falls outside the laboratory reference range and meets the clinical significance criteria for liver and kidney tests as well as for hematology and clinical chemistry, etc. (i.e. AST and/or ALT > 2 x ULN).

This does not apply to any abnormal laboratory result which falls outside the laboratory reference range but which does not meet the clinical significance criteria or those which are a result of an AE which has already been reported.

Any laboratory result abnormality fulfilling the criteria for a serious adverse event (SAE) should be reported as such, in addition to being reported as an AE in the study record.

11.2 Treatment and Follow-Up Of AEs And Laboratory Abnormalities

11.2.1 Treatment and Follow-up of AEs

AEs, especially those for which the relationship to the investigational product is suspected, should be followed up until they have returned to baseline status or stabilized.

If after follow-up, return to baseline status or stabilization cannot be established, an explanation should be recorded in the study record.

11.2.2 Treatment and Follow-up of Laboratory Abnormalities

In the event of participant-initiated withdrawal or clinically significant unexplained abnormal laboratory test values, the participant will be withdrawn from the treatment and will remain in the study and be required to attend all remaining study visits as part of a safety arm.

11.3 Reporting of SAEs And Unexpected Adverse Reactions

The qualified investigator will be responsible for classification of an AE as an SAE within 24 hours of notification. Causality should be signed off by the qualified investigator prior to reporting to ethics and regulatory bodies. Notification of any serious adverse events must be made in writing to the study sponsor. The IRB will be notified of all product related SAEs and unexpected adverse reactions. All blinded SAE's or unblinded-participant-on-active product SAE's will be reported to the Therapeutics Products Directorate (TPD) in an expedited manner.

KGK Science must notify the TPD of all blinded or unblinded-participant-on-active product serious adverse events and reactions as follows:

If it is neither fatal or life threatening, within 15 calendar days after the day on which the sponsor becomes aware of the information; and

If it is fatal or life threatening, must be reported as soon as possible, but not later than seven (7) days after the day on which the sponsor becomes aware of the information.

12 STATISTICAL EVALUATION

12.1 Determination of Sample Size

With a total sample size of 100 (50 per group; 40 completers), it is possible to detect a difference in mean change in percent body fat or body fat mass of 0.76% or 0.79 kg respectively, in a parallel design at a two-sided unadjusted 5% significance level, 80% power and 20% attrition rate. This calculation is based on standard deviations of 1.19% and 1.24 kg for percent body fat and body fat mass respectively, estimated from a study by Minami et al (2018).

12.2 Analysis Plan

The **Safety Population** will consist of all participants who received any amount of either product, and on whom any post-randomization safety information is available.

The **Intent-to-Treat (ITT) Population** consists of all participants who received either product and on whom any post-randomization efficacy information is available.

The **Per Protocol (PP) Population** consists of all participants who consumed at least 80% of treatment or placebo doses, do not have any major protocol violations and complete all study visits and procedures connected with measurement of the primary variable.

12.3 Statistical Analysis Plan

For each continuous endpoint including percent body fat, body fat mass, visceral fat area, weight, BMI, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, waist circumference, and hip circumference, fasting blood glucose, HbA1c, fasting insulin levels, AST, ALT, GGT, ALP, a summary table will be prepared with a variety of summary statistics including mean, standard deviation, median, min-max range and number of participants analyzed for each time point-treatment pairing. These data will be classified in subgroups based on the similarity of microbiota composition.

To account for any a priori differences between groups, the similar summary statistics of the changes from baseline will also be provided. Mean values will be displayed as graphs, with a separate line for each product, and error bars indicating ± SEM. Mean changes from baseline will also be graphed similarly. Changes in continuous endpoints from screening/baseline will be calculated as:

$$Change\ to\ V_i = Value\ at\ V_i - Value\ at\ V_{screening/baseline}$$

Continuous variables will be tested for normality and log-normality using histograms, box plots and Q-Q plots. Log-normally distributed variables will be analyzed in the logarithmic domain. Summary statistics will be provided as non-transformed values. Intractably non-normal variables will be analyzed by appropriate non-parametric tests.

The primary endpoint - change in fat loss from baseline (% or g), as assessed by Dual-Energy X-Ray Absorptiometry (DXA), between *B. breve* and placebo after 12 weeks of supplementation - will be assessed for between-group differences using ANCOVA, with the baseline measurements as covariate.

Continuous secondary endpoints will be tested for between-group differences using repeated measures linear mixed-effects ANCOVA. This will allow for the random effects associated with each subject to be accounted for. The dependent variable will be the change at each visit, the fixed effects will be the study group * visit number, and the covariate will be the dependant variable at baseline (Day 0).

Baseline values for continuous variables will be tested for between-group differences using the independent Student's t-test. Within-group differences will be assessed using the Student's paired t-test or, in the case of intractable non-normality, the Wilcoxon sign rank test. The frequency of BMs will be calculated as the average number of BMs per day over the 7 days prior to baseline and 7 days prior to week 6 and week 12.

For categorical variables, counts and percentages will be presented. The denominator for each percentage will be the number of subjects within the study group for that visit/week unless otherwise specified. Possible differences between groups will be assessed by using the two-tailed Chi-squared or Fisher's exact test, as appropriate.

Microbiota analysis will be performed at Morinaga Milk Co., Ltd. The subgroups will be determined based on the similarity of microbiota composition by hierarchical clustering. These subgroups will be defined before analyzing week 12 data.

All missing efficacy/effectiveness values analysis will be imputed using the most recent previously-available value (LOCF, or "last-observation-carried-forward" imputation) or multiple imputation. No imputation will be performed for missing values of safety variables.

All statistical analysis will be completed using R Statistical Software version 3.5.3 (R Core Team, 2019) for Microsoft Windows or SAS software, Copyright © 2019 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

12.3.1 Premature Discontinuation Description

For each premature discontinuation, the following parameters will be listed: participant number, dates of start and end of treatment, and the reason of premature discontinuation.

12.3.2 Safety

For adverse events, a descriptive analysis will be given. Adverse events will be presented in a frequency table by category and treatment. Furthermore, description, frequency, severity, and causality will be reported for each adverse event.

Continuous safety parameters (e.g. hematology, clinical chemistry, heart rate and blood pressure, urine specific gravity) will be summarized using a table including mean, standard deviation, median, minimum value, and maximum value for each measurement point. These will be compared to normal clinical ranges and any abnormalities will be discussed.

Categorical safety parameters (e.g. incidence of adverse events and urinalysis (microscopy, glucose, ketones, blood, protein, colour)) will be summarized as counts and percentages. The denominator for each percentage will be the number of subjects within the study group for that visit/week unless otherwise specified. Possible differences between groups will be assessed by using the two-tailed Chi-squared or Fisher's exact test, as appropriate.

12.4 Protocol Deviation Description

Protocol deviations will be listed in the final study report.

12.5 Protocol Amendments

Once the protocol has been approved by the IRB and Health Canada, any changes to the protocol must be documented in the form of an amendment. All amendments will be documented in the final study report.

13 DATA COLLECTION AND STORAGE

All data collection and record storage will be done in compliance with ICH GCP Guidelines and applicable local regulatory guidelines.

14 ETHICAL ASPECTS OF THE STUDY

This study will be conducted with the highest respect for the individual participants (i.e., participants) according to the protocol, the ethical principles that have their origin in the Declaration of Helsinki, and the ICH Harmonised Tripartite Guideline for GCP.

14.1 IRB Approval

KGK Science Inc. will supply relevant documents for submission to an IRB for the protocol's review and approval. The following must be submitted to the IRB: this protocol, a copy of the informed consent form, and, if applicable, volunteer recruitment materials and/or advertisements and other documents required by all applicable laws and regulations. The IRB's written approval of the protocol and volunteer informed consent must be obtained before commencement of the study. The IRB approval must refer to the study by exact protocol title, number, and version date; identify versions of other documents (e.g., informed consent form) reviewed; and state the approval date.

KGK must adhere to all requirements stipulated by the IRB. This may include notification to the IRB regarding protocol amendments, updates to the informed consent form, recruitment materials intended for viewing by volunteers, local safety reporting requirements and submission of the investigator's annual/final status report to the IRB.

14.2 Volunteer Information and Informed Consent

Written consent documents will embody the elements of informed consent as described in the declaration of Helsinki and the ICH Guidelines for GCP and will be in accordance with all applicable laws and regulations. The informed consent form describes the planned and permitted uses, transfers, and disclosures of the volunteer's personal and personal health information for purposes of conducting the study. The informed consent form further explains the nature of the study, its objectives, and potential risks and benefits, as well as the date informed consent is obtained. The informed consent form will detail the requirements of the volunteer and the fact that he or she is free to withdraw at any time without giving a reason and without prejudice to his or her further medical care.

14.3 Potential Risks and Procedures to Minimize Risk

All potential risks are disclosed to study participants prior to their participation. The potential risks associated with this study include venipuncture and the associated risks. Risks associated with venipuncture include pain, bruising, and infection at the site. Alcohol swabs and proper venipuncture procedures will be followed to minimize the risk of infection.

15 QUALITY ASSURANCE AND QUALITY CONTROL

15.1 Auditing

All materials used in clinical studies are subjected to quality control. Quality assurance audits may be performed by the sponsor or any health authority during the course of the study or after its completion.

The Investigator agrees to comply with the sponsor and regulatory requirements in terms of auditing of the study. This includes access to the source documents for source data verification.

15.2 Monitoring

An initiation meeting will be conducted by the sponsor or an approved representative (CRO). At this meeting, the protocol and logistical aspects of the study will be reviewed with the Investigator and all study staff.

Source documents will be reviewed to ensure that all items have been completed and that the data provided are accurate and obtained in the manner specified in the protocol. The participant files will be reviewed to confirm that:

1. Informed consent was obtained and documented
2. Enrolled participants fulfilled all inclusion criteria and did not meet any exclusion criteria;
3. AE/SAE reporting has been performed as applicable
4. Study visits have been conducted as per protocol and information has been recorded in the appropriate place in the source document
5. The study product is being stored correctly and an accurate record of its dispensation to the study participants is being maintained (accountability)

Incorrect, inappropriate, or illegible entries in the participant files will be returned to the Investigator or designee for correction. No data disclosing the identity of participants will leave the study center. The Investigator and any designees will maintain confidentiality of all participant records.

The Investigator will permit trial-related monitoring, audits, IRB/IEC review, and regulatory inspections and will allow direct access to source data and documents for these purposes.

15.3 Data Management

Data required for the analysis will be acquired from source documentation (including laboratory reports) and entered into Open Clinica Enterprise Version study instance designed specifically for this study. The two instances for the database would be created, test instance and production instance. A UAT (User Acceptance Testing) of the database would be performed in the test instance and then moved to the production instance. A password protected user id's will be created which would give access to the limited authorized personnel. Only properly trained Data management staff will be granted access to perform database designing, according to SOP - Designing Database in Open Clinica Enterprise Version and SOP - Creating user in Open Clinica Enterprise Version. A study-specific Data Management Plan will be generated after the finalization of the database.

The standard data validation and edit checks would be performed on the production instance of the study by designing study specific rules and restrictions as defined in the eCRF. The discrepancies will be queried and managed according to the SOP - Discrepancy Management SOP in Open Clinica Enterprise Version. Data tables will be created, queried and exported during and at the end of study using PostgreSQL tool (pgadminIII 9.5) and MS Access.

For Statistical analysis, the validated soft-lock copy of the blinded study database will be sent to the Statistician to perform the analysis. The study database would be a read-only file to ascertain changes in the data are not made during or after the analysis.

High safety standards for the transfer and storage of study data are guaranteed by the use of technologies such as password protection, firewalls and periodic backup to protect stored data.

All study data is archived for a period not less than 25 years from the date of completion of the study in accordance with Health Canada regulatory requirements.

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17 APPENDICES

17.1 Appendix I: Probiotic and Prebiotic Foods to Avoid

Dietary Guidelines

Please use the following guidelines to help you make meal and snack choices between study visits that are appropriate for the study.

****** Please refrain from ingesting any foods or beverages with live bacteria or yeast present during the course of the trial. ******

Supplements to Avoid:

Probiotic supplements – any food, capsule or orally administered product containing live bacteria or yeast, often labeled as bacteria or yeast fermentation products (such as: *Lactobacillus, Bifidobacteria, Bacillus, saccharomyces*)

Prebiotic supplements – any orally administered product with the specific function of increasing bacterial populations.

Foods to Avoid:

- Yoghurt
- Naturally fermented cabbage (including but not limited to sauerkraut, kimchi)
- Naturally fermented pickles, cucumbers, and other vegetables
- Fermented soy products (including but not limited to tempeh, miso, natto)
- Uncooked buttermilk products
- Soft cheeses, live culture cheeses (including but not limited to blue cheese, gouda, goat cheese)
- Cold or uncooked sausage products
- Foods marketed as “Probiotic” or “Live Culture”

Beverages to Avoid:

- Kefir
- Kombucha
- Apple cider vinegar
- Lambic beers
- Sour ales
- Wheat beers
- Other beverages labeled as probiotic

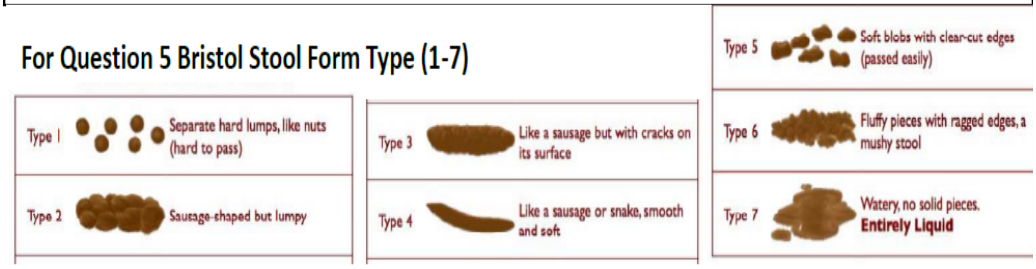
Foods to Limit: (~2 servings per week)

- Onions, leeks, garlic
- Asparagus, artichokes, chicory root, dandelion greens

17.2 Appendix II: Daily Bowel Habits Diary with Bristol Stool Scale

Daily Bowel Habits Diary with Bristol Stool Scale will be completed every day by the participants.

Date:						
Comments/Therapies/Medication (dose, frequency, reason for use)/Changes in Health:						
For each bowel movement you experienced today record the time (in 24-hour format) for question 1 and circle Yes or No (Y/N) for questions 2-7. **For question 5 please refer to the Bristol Stool Scale and record the type that best describes the bowel movement.** **If you do not have any bowel movements, circle 0 and complete question 2, 6 and 7.**						
	Bowel Movement Number *** If required, additional movements and details can be added to the back of the diary**					
	0	1	2	3	4	**5**
1. Time of Bowel Movement (hh:mm)	N/A	___ : ___	___ : ___	___ : ___	___ : ___	**___ : ___**
2. Straining to start defecation?	Y / N or N/A	Y / N	Y / N	Y / N	Y / N	**Y / N**
3. Straining to stop defecation?	N/A	Y / N	Y / N	Y / N	Y / N	**Y / N**
4. Feeling of incomplete defecation?	N/A	Y / N	Y / N	Y / N	Y / N	**Y / N**
5. Bristol Stool Form Type (1-7) see below?	N/A					
6. Were laxatives, enemas or suppositories used in the 24 hours prior to the bowel movement?	Y / N	Y / N	Y / N	Y / N	Y / N	**Y / N**
7. For Females: Do you feel that your bowel movements today were affected by your menstrual cycle? Y / N						



17.3 Appendix III: Exercise Guidelines

Please use the following guidelines to help you select the type of exercise that is appropriate for this study

****** Please ensure that you exercise 3 times per week for 20 minutes at a <u>moderate intensity</u> (can be in bouts of 5-10 minutes)******

Examples of moderate and high-intensity exercise

Moderate-intensity Physical Activity Activities elevate the heart rate and cause light sweating after about 10 minutes. Breathing is heavier than normal, but you can carry a conversation.	High-intensity Physical Activity Activities elevate the heart rate substantially and cause significant sweating. You cannot carry a conversation due to heavy breathing.
• Brisk walking • Bicycling (easy pace) • Light to moderate workouts on gym equipment • Water aerobics • Swimming (leisure pace) • Carrying moderate loads • Participating in active games with children (eg. Hopscotch, dodgeball) • Dancing (social)	• Running • Bicycling (brisk pace) • Swimming (fast pace) • Carrying heavy loads • Playing competitive sports • Cross-country skiing • Dancing (competitive)