Statistical Analysis Plan (SAP)

Protocol Title:	A randomized, double-blind, placebo-controlled study to investigate the effects of a Bifidobacterium breve strain on fat loss in healthy adults
SAP Date:	November 6, 2023
SAP Version:	1
Study Design:	Randomized, double-blind, placebo-controlled, 2-arm parallel study
Sponsor:	
Sponsor Contact:	
CRO:	
Medical Director:	

1. SIGNATURES

I have read and approve of this statistical analysis plan for use in this study.

STATISTICIAN

	Date

ASSISTANT SCIENTIFIC DIRECTOR

	Date

SPONSOR

	Date

Table of Contents

2. OVERVIEW AND ENDPOINTS

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.

2.1 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

2.2 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)

2.3 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

3. STUDY POPULATION

A total of three populations are defined for all summaries and analyses. Subjects who satisfy the inclusion/exclusion criteria will be classified in the designated populations:

- **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** will consist of all participants who received either product and on whom any post-randomization efficacy information is available.

- **The Per Protocol (PP) Population** will consist 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.

4. STATISTICAL METHODS

4.1 Demographics and Baseline Characteristics

Summary statistics, including mean, standard deviation, median, minimum, and maximum on continuous demographic and baseline characteristic variables will be obtained for the ITT and PP populations. Categorical demographic and baseline variables will be summarized with frequencies and percentages for the ITT and PP populations.

Continuous variables will be evaluated for normality using Q-Q plots. Two sample t-test will be used to examine differences between the two study arms (B. breve vs. placebo) if the normality assumption is satisfied, and Wilcoxon rank sum test will be used otherwise. Categorical variable distribution between study arms will be assessed by Chi-squared or Fisher's exact test, as appropriate.

The demographic and baseline variables may include but not limited to:

- Age (years)
- Gender (male, female)
- Race
- Ethnicity

4.2 Compliance

The IP compliance will be assessed by counting the returned unused test product at each visit. The IP 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.

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

Possible differences in the IP compliance between B. breve and placebo groups will be assessed by two-sample t-test if the normality assumption is satisfied, and Wilcoxon rank sum test will be used otherwise.

Compliance will also be assessed for exercise.

4.3 Protocol Deviations

Protocol deviations will be listed in the final study report.

4.4 Level of significance

The significance level for this study will be $p \leq 0.05$ (5%).

5. EFFECTIVENESS/ EFFICACY ANALYSIS

5.1 Primary outcomes

Primary Outcomes	Hypothesis
1- The difference in change in fat loss from baseline (% or g), as assessed by DXA, between B. breve and placebo after 12 weeks of supplementation.	H0: There is no difference in mean change in fat loss, as assessed by DXA, from baseline at week 12 between B. breve and placebo

Summary statistics including number of participants, mean, median, standard deviation, minimum, and maximum will be obtained for baseline and week 12 visits. Similar summary statistics will be obtained for the change from baseline to week 12. 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.

The primary outcome will be assessed using ANCOVA, with the baseline measurements as covariate and group as the factor variable. The dependent variable will be the change from baseline at week 12.
Q-Q plots and residuals versus fitted values plots will be generated for the evaluation of the model adequacy. If the residual plots show violation of the model assumptions, log or other transformation of the dependent variable will be performed.

Between group differences will be assessed by two-sample t- test. If the outcome is not normally distributed, then Wilcoxon rank sum test will be used.

Within group differences will be assessed by paired t-test if the outcome is normally distributed and Wilcoxon signed rank test otherwise. P-values and 95% confidence intervals will be calculated.

5.2 Secondary Outcomes

Secondary Outcomes	Hypothesis
1. The difference in change from baseline between B. breve and placebo in body composition (weight, BMI, android/gynoid fat ratio, and muscle mass (% or g)) as assessed by DXA after 12 weeks of supplementation	H01.1: There is no difference in mean change in body composition (weight, BMI, android/gynoid fat ratio, and muscle mass (% or g) as assessed by DXA from baseline at week 12 between B. breve and placebo
2. The difference in change from baseline between B. breve and placebo in waist circumference, hip circumference, and	H02.1: There is no difference in mean change in waist circumference, hip circumference, and waist/hip

waist/hip circumference ratio after 6 and 12 weeks of supplementation	circumference ratio from baseline at week 6 between B. breve and placebo H02.2 There is no difference in mean change in waist circumference, hip circumference, and waist/hip circumference ratio from baseline at week 12 between B. breve and placebo
3. The difference in change from baseline between B. breve and placebo in Lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides) after 12 weeks of supplementation	H03.1: There is no difference in mean change in Lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides) from baseline at week 12 between B. breve and placebo
4. The difference in change from baseline between B. breve and placebo in biomarkers of glycemic control (fasting blood glucose, HbA1c, fasting insulin levels) after 12 weeks of supplementation	H04.1: There is no difference in mean change in biomarkers of glycemic control (fasting blood glucose, HbA1c, fasting insulin levels) from baseline at week 12 between B. breve and placebo
5. The difference in change from baseline between B. breve and placebo in liver markers (AST, ALT, GGT, ALP) after 12 weeks of supplementation	H05.1: There is no difference in mean change in liver markers (AST, ALT, GGT, ALP) from baseline at week 12 between B. breve and placebo
6. The difference in change from baseline between B. breve and placebo in the frequency of bowel movements after 6 and 12 weeks of supplementation	H06.1: There is no difference in mean change in the frequency of bowel movements from baseline at week 6 between B. breve and placebo H06.2: There is no difference in mean change in the frequency of bowel movements from baseline at week 12 between B. breve and placebo

Summary statistics including number of participants, mean, median, standard deviation, minimum, and maximum will be obtained for each visit. Similar summary statistics will be obtained for each available change from baseline to each subsequent visit. 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.

Continuous secondary outcomes #2 and # 6 will be assessed using linear mixed effects ANCOVA, the fixed effects will include the baseline measurements as covariate and group*visit as the factor variables. The dependent variable will be the change at each visit.

Continuous secondary outcomes #1, #3, #4, and #5 will be assessed using ANCOVA, with the baseline measurements as covariate and group as the factor variable. The dependent variable will be the change from baseline at week 12.

Q-Q plots and residuals versus fitted values plots will be generated for the evaluation of the model adequacy. If the residual plots show violation of the model assumptions, log or other transformation of the dependent variable will be performed.
Between group differences will be assessed by two-sample t- test. If the outcome is not normally distributed, then Wilcoxon rank sum test will be used.

Within group differences will be assessed by paired t-test if the outcome is normally distributed and Wilcoxon signed rank test otherwise. P-values and 95% confidence intervals will be calculated.

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.

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.

[The microbiota analysis outcomes (#7 and #8) will be covered in a separate SAP].

5.3 Exploratory Outcomes

Exploratory Outcomes	Hypothesis
9. Total body fat (%) change during the run-in period as assessed by BIA	H09.1: There is no difference in mean change in total body fat (%) during the run-in period as assessed by BIA between B. breve and placebo

10. Comparison of total body fat (%) as assessed by BIA and DXA at baseline	H010.1: There is no difference in mean in total body fat (%) as assessed by BIA and DXA at baseline between B. breve and placebo

Exploratory outcomes differences will be assessed by two-sample t- test. If the outcome is not normally distributed, then Wilcoxon rank sum test will be used.

6. SAFETY ANALYSIS

6.1 Safety Outcomes

For continuous safety parameters, summary statistics including number of participants, mean, median, standard deviation, minimum, and maximum will be obtained for baseline and Visit 4. Similar summary statistics will be obtained for the changes from baseline.

The outcome will be assessed for normality using Q-Q plots. If the outcome is normally distributed, then between group differences will be assessed by two-sample t-test. If the outcome is not normally distributed, then Wilcoxon rank sum test will be used. Within group differences will be assessed by paired t-test if the outcome is normally distributed and Wilcoxon signed rank test otherwise. P-values and 95% confidence intervals will be calculated.

Categorical safety parameters 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.

6.2 Adverse Events

A descriptive analysis will be provided for pre-emergent and post-emergent AEs. AEs will be presented along with the system organ class, preferred term, and lower-level term. Furthermore, description, frequency, severity, and relationship to study product will be reported for each AE.

7. MISSING 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 techniques will be performed for missing values of safety variables.

Lab measurements that are below or above the threshold of quantification, will be imputed with that threshold value.