IRB - **Pro00070958**

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STARS: **S**ynbiotic **T**o **A**ttenuate **R**esorption of the **S**keleton

A Randomized, Double-Blind, Placebo-Controlled, Clinical Food Trial of Probiotic/Prebiotic Medical Food for the Dietary Management of Age Related Bone Loss

STATISTICAL ANALYSIS PLAN

VERSION 1.0

4 NOVEMBER 2025

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List of Abbreviations

AE	Adverse Event
CI	Confidence interval
CRF	Case Report Form
DXA	Dual-energy X-ray absorptiometry
SAE	Serious Adverse Event
qCT	Quantitative Computed Tomography

1 Introduction

This document provides detail to elaborate the pre-planned statistical analysis described in protocol (Version 12.0, dated 4 November 2025) for study Pro00070958 - **S**ynbiotic **T**o **A**ttenuate **R**esorption of the **S**keleton (**STARS**), *A Randomized, Double-Blind, Placebo-Controlled, Clinical Food Trial of Probiotic/Prebiotic Medical Food for the Dietary Management of Age-Related Bone Loss.* This is a single-site, double-blind, randomized placebo-controlled trial (**RCT**).

2 Study Background and Rationale

2.1 The Gut Microbiome and Maintenance of Bone Mass

The human gut microbiome harbors a diverse set of microbial species that have coevolved with humans over millions of years.[1] The composition of this ecosystem depends on both intrinsic and extrinsic factors, including host genetics and diet.[2,3] The use of health promoting probiotic microbes (live strains of bacteria and fungi that are orally administered and function along the gastrointestinal tract) and prebiotic dietary fibers (indigestible dietary fibers that specific commensal bacteria consume for fuel and metabolize into beneficial biproducts) have been well described in the scientific literature. Multiple studies have demonstrated that commercially available probiotic bacteria are protective in ovariectomy-induced bone loss, the gold standard animal model of postmenopausal osteoporosis.[4-10] A number of double-blind, placebo-controlled clinical trials have demonstrated significant efficacy of commercial probiotics in maintaining bone mass in postmenopausal women.[11-13]

2.2 SBD111

SBD111 is a medical food consisting of a *Lactobacillus brevis*, a *Leuconostoc mesenteroides*, a *Lactobacillus plantarum*, and a *Pichia kudriavzevii.* It is an orally delivered capsule containing a mixture of live microbial probiotic strains (consisting of *Lactobacillus plantarum, Lactobacillus brevis, Leuconostoc mesenteroides, and Pichia kudriavzevii*) with natural prebiotics and inert formulation ingredients. SBD111 has demonstrated preclinical efficacy and dose-response behavior in maintaining lumbar spine bone mineral density (BMD) in estrogen-depleted female mice as well as preliminary safety in an additional rodent model.

2.3 Placebo comparator

The matching placebo (**Placebo** or **Control**) consists of rice flour. The placebo formulation has the same appearance of Active Product including packaging and administration.

2.4 Study rationale

The hypothesis is that the orally dosed *synbiotic* (prebiotic and probiotic) SBD111 will aid in the dietary management of bone loss women at least 60 years of age.

3 Summary of Design Features

This RCT will enroll approximately 220 participants. The visit schedule is described at length in the trial protocol. Outcomes will be evaluated at baseline, and at 9 months & 18 months post-randomization.

3.1 Ascertainment of Study Outcomes

The measurement protocol is described in the protocol in detail. Briefly:

Safety will be measured by incidence of adverse events (AE) and serious adverse events (SAE) over the study duration. Details are provided in Section 6.1 and in the trial protocol.

Efficacy outcomes defined in Section 6 will be computed from measures acquired as follows:

Bone Mineral Density (BMD) at the proximal femur and lumbar spine (L1-L4) will be measured by dual-energy x-ray absorptiometry (DXA) at baseline, 9 months, and 18 months. Proportionate change in BMD at each follow-up time, relative to baseline, will be computed for each participant.

Vertebral compressive strength Quantitative Computed Tomography (**qCT**) scans acquired at baseline and 18 months will be used to estimate vertebral compressive bone strength via a biomechanical computed tomography (BCT) algorithm.

Volumetric BMD (vBMD) will be quantified by BCT at T12-L3.

Biomarkers of bone resorption (β-CTX) and formation (P1NP) will be measured in blood at baseline, 9 months and 18 months.

Inflammatory cytokines (TNFα and IL-17) will be measured at baseline and 18 months.

Gut microbiome will be measured using whole metagenomics sequencing (WMS) from stool samples at baseline and 18 months. Potential future investigations may also include utilizing the assays for metatranscriptomics.

4 Overall Study and Analysis Timeline

Measurements will be taken at a screening visit, a baseline assessment, and 9 and 18 months post baseline. Select outcomes measures (see above) will be excluded from the 9-month measurement protocol.

4.1 Enrollment criteria

Eligibility will be determined by means of telephone and in-person screening.

To be eligible for *inclusion*, potential subjects will:

- Execute written informed consent to study enrollment, randomization and other procedures
- Be available throughout entire study period and state willingness to fulfill all details of the protocol
- Be a woman at least 60 years of age
- Have dual energy X-ray absorptiometry-derived Bone Mineral Density T-score > -2.5 at the lumbar spine (L1-L4) and femoral neck. Women with a BMD T score ≤ -2.5 (i.e. women with low BMD indicating osteoporosis) will be considered if they have decided not to accept the standard of care with use of osteoporosis medications for the entire duration of their participation in the study.
- Have 25-hydroxy vitamin D ≥ 20 ng/mL
- Have normal renal function (eGFR > 50 ml/min)
- Have chosen not to accept the standard of care with use of osteoporosis medications
- Be willing to comply with procedures and report on compliance at regular intervals

Potential participants will be *excluded* from participation if any of the following is present:

- BMI greater than 40 kg/m^2
- Regular consumption of dietary supplements or unwillingness to avoid supplements during the study
- Known or suspected allergies to probiotics, rice, edible fruit extract or berries
- History of drug or alcohol misuse
- Participation in another clinical trial with similar aims within the past six months
- Seated blood pressure greater than 160 mmHg

- Morbidities or medication or laxative use as detailed in the trial protocol
- Other disqualifying circumstances as adjudicated by a trial physician or investigator

4.2 Randomization criteria

Participants will be eligible for *randomization* if they meet enrollment criteria and consent to randomization.

4.3 Randomization procedure

Eligible participants will be then allocated in permuted blocks to the two study arms in a 1-1 ratio by a computer algorithm.

5 Study Objectives

5.1 Primary Objective

To determine the effect of 18 months of daily intake of SBD111 on the primary outcome of lumbar spine DXA-BMD and secondary outcomes (BCT-derived vertebral compressive strength, vBMD, and markers of bone turnover) in women.

5.2 Secondary Objectives

To determine the effect of 18 months of daily intake of SBD111 on markers of inflammation and gut microbiome function (secondary outcomes) in women. These include assessments of secondary outcomes IL-17 and TNF-α.

6 Endpoints

6.1 Safety Endpoints

Safety will be assessed using the **incidence** (total count and number of participants experiencing) of AE and SAE. Adverse events (again both total incidence and number of participants experiencing one or more) will also be characterized according to **severity** (mild, moderate, severe) and probability of **relatedness** (not related, remote, possible, probable, highly probable) to trial or the intervention procedures. Time since product administration and time to resolution of adverse events will be computed and displayed.

6.2 Primary Efficacy Endpoint

The **primary efficacy outcome measure** is the proportionate change (relative to baseline) in BMD, as defined in Section 3.1, at the lumbar spine (L1-L4).

6.3 Secondary Efficacy Endpoints

Secondary efficacy outcome measures will include percent change in vertebral compressive strength, vBMD, β-CTX, P1NP, TNFα, IL-17, and gut microbiome function as defined in Section 3.1, as well as proportionate change in BMD at the proximal femur.

6.4 Participant-level assessment of change in efficacy endpoints

Proportionate nine (as relevant) and 18-month changes in endpoints will be computed at the level of the participant. Where analyses consider percent change, proportionate or percent increase (positive change) or decrease (negative change) will be expressed relative to the baseline value.

6.5 Adherence to test article administration

Adherence will be assessed at the participant level as the proportion of scheduled intake of SBD111 completed. Adherence of 75% may be used to determine which participants are eligible for a "per protocol" analysis. A secondary analysis of efficacy of the intervention will employ adjustment for this measure.

7 Analysis Populations

Approximately 220 women will be enrolled.

The **efficacy and safety population** will consist of all individuals meeting inclusion and exclusion criteria and who are randomized. The **per protocol population** will consist of participants achieving the 75% adherence threshold described in Section 6.5.

7.1 Primary Efficacy Analysis

Primary efficacy analysis will make use of the efficacy and safety population.

7.2 Secondary Efficacy Analysis Population

Secondary efficacy analysis will make use of the efficacy and safety population

7.3 Other Efficacy Analyses

Analyses of other efficacy endpoints will make use of the efficacy and safety population, with the exception of "per protocol" analyses, which will make use of the per protocol population.

7.4 Safety Analysis Population

Safety analyses will make use of the efficacy and safety population.

7.5 Prespecified Analytic Subgroups

Supporting assessments of the primary and secondary efficacy outcome measures will group participants by:

- Age (< 70 years vs. 70+ years)
- Osteopenic or osteoporotic (T-score < -1) vs. other
- BMI > 30 kg/m^2 vs. other

8 Analysis Conventions

8.1 Assessment of Uncertainty and Statistical Significance

Point estimates obtained in analyses described below will accompanied by 95% confidence interval estimates of effects. Limited hypothesis testing will be conducted at the 0.05 level.

8.2 Control of Type-I Error Probability

There will be no adjustment for multiplicity. Analyses of primary and secondary endpoints are prespecified and mutually reinforcing. Findings of difference and no difference will be publicized equally and treated together in presentation.

8.3 Interim Analysis

No interim analysis of efficacy endpoints is planned.

8.4 Missing Data

Regression models supporting the primary and secondary analyses of efficacy endpoints will be by linear and generalized linear models as well as their mixed-effects counterparts (see below), which will make use of all available data on the efficacy and safety population. Models employing this convention have shown similar performance to those employing multiple imputation for efficiency and for coverage of interval estimates.[14]

8.5 Target Estimands

Analyses will develop model-based point and robust interval estimates of mean differences in primary and secondary endpoints attributable to application of intervention to 9 months (as relevant) and 18 months.

8.6 Quality Assurance

As appropriate, analyses may exclude anomalous imaging readings or spurious values resulting from measurement artifacts, injuries, or the presence of other interference.

8.7 Sample Size Justification

Sample size is motivated by the stipulation of obtaining 80% power to detect a standardized difference of 0.40 between groups on each efficacy endpoint measured at 18 months, which is anticipated to correspond to a 1.2% in 18-month percent change in BMD between intervention and control at 18 months. This requires evaluable data of 99 participants per arm, suggesting total enrollment of 220 individuals under the assumption of 10% cumulative missingness and attrition. Owing to serial correlation in outcomes measured at 9 months, we anticipate somewhat greater power for detection of clinically significant differences on those endpoints.

9 Data Analysis

9.1 Description of sample

Age, height, weight, medical history, baseline endpoint measures and other variables will be summarized using relevant descriptive statistics.

9.2 Analysis of safety endpoints

Total incidence of adverse events, as well as number of participants experiencing one or more events, will be presented as counts (median and range) and counts (percent), respectively. Where appropriate, Wilcoxon tests or Poisson regression estimates of number of events per subject per time may be utilized to derive statistical significance of differences in the incidence of events, and chi-square or Fisher’s exact tests of the number of individuals with one or more event may be provided, again where appropriate and supported by the total number of events and participants with at least one event.

9.3 Analysis of primary efficacy endpoint

Primary analysis of the primary outcome will develop a mixed-effects regression model of the percent change in BMD at 9 and 18 months simultaneously, with random effects for participant, controlling for baseline BMD. The primary target of estimation will be the between-arm (absolute) difference in percent change in BMD at 18 months, estimated by a treatment contrast and associated 95% confidence interval. For outcomes not collected at 9 months, linear regression model of 18-month outcomes will be employed. As noted above, robust standard error estimates will be employed for computation of confidence interval estimates and determinations of statistical significance.

9.4 Analysis of secondary efficacy endpoints

Analysis of secondary endpoints will proceed in parallel fashion to those of the primary endpoint. For analysis of the gut microbiome a) taxa will be tested for differences in log relative abundance; b) taxa will also be tested for differences in prevalence using logistic regression; c) all regressions will use Benjamini-Hochberg False Discovery Rate (FDR) adjusted P value. Plots and tables will be reported out for only the top 20 most significantly different pathways in each analysis to keep the reporting manageable.

9.5 Secondary analysis of primary and secondary endpoints

As appropriate, secondary analyses controlling for potential baseline imbalances between arms (as determined by inspection and investigator assessment of clinical importance) may be conducted to support and clarify primary analyses. Additional analyses will consider robustness of results to exclusion of measurements occurring after introduction of antibiotics post-randomization.

9.5.1 Pre-planned subgroup analyses

Prespecified sample subgroups (see above) describe subsets of the efficacy population. Efficacy endpoints will be re-analyzed restricting attention to participants in each pre-planned subgroup, applying the methods described above for each outcome measure. Only primary analyses for each endpoint will be reproduced for planned subgroups. Where appropriate, limited hypothesis testing of effect modification by subgroup will be considered.

9.5.2 Sensitivity Studies

Sensitivity analyses may develop model-based estimates taking account the presence of intercurrent events and illnesses recorded during the intervention period.

10 Summary of Changes

Procedures described above are consistent with those described in the study protocol Version 12.0, dated 4 November2025. Where statistical procedures described here differ from those in the protocol, those described here will be considered controlling.

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