Statistical Analysis Plan for the RAMP Study

*R*ejuvenation of the *A*ging *M*icrobiota with *P*rebiotics

A collaboration between Abbott Nutrition R&D and the Sonnenburg and Gardner Labs at Stanford University

Clinical Trial Identifier: NCT03690999

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This document specifies a statistical analysis plan for data derived from the RAMP study: a randomized, double-blind, prospective, placebo controlled human intervention study detailed in Figure 1 below.

Purpose

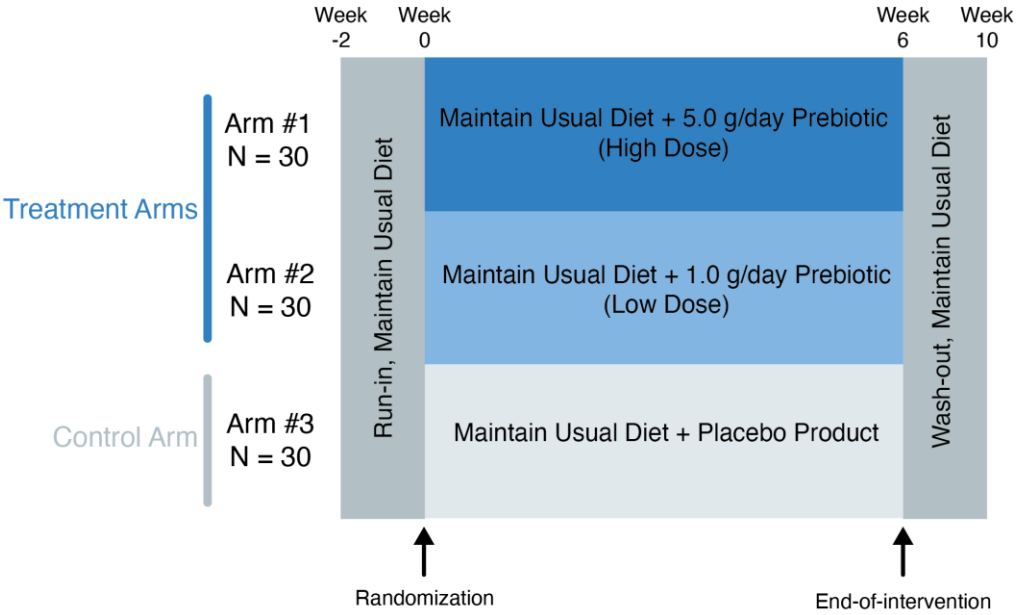
The objective of this study is to define the impact of a prebiotic supplement on microbiome, immune system, and metabolic status in older adults. This study will determine the degree to which a prebiotic supplement can 1) regulate immune status and function including reducing chronic, systemic inflammation as assessed by high dimensional immune profiling, 2) alter microbiota composition and function, 3) impact microbiota metabolites, and 4) alter metabolic markers.

Background

An individual's immune and metabolic status is coupled to components of diet that interact directly with the host (i.e., are absorbed in the small intestine as part of classic digestion) or via gut microbial metabolism. Complex carbohydrates that are not digested by human enzymes may influence host biology by impacting microbiota composition and function, or act in a yet-unknown microbiota-independent manner. Prebiotics offer a promising and safe route to influence host health, via microbiota-independent or -dependent mechanisms. However, it remains largely unknown to what extent human immune function and metabolism can be modulated by prebiotics.

Figure 1. Study Design.

Arms and Interventions



Subjects were randomized to one of three arms: 1) High Dose Prebiotic, 2) Low Dose Prebiotic, and 3) placebo control. Subjects were monitored for 2 weeks prior to the start of the intervention during a "run-in" period, then consumed their prebiotic product or placebo product for 6 weeks and then were monitored again for 4 weeks during a "wash-out" period.

Sample Collection Timeline

	Baseline 1		Baseline 2			Mid-intervention			End-of-intervention	Washout
Week	-2	-1	0	1	2	3	4	5	6	10
Stool & Blood Sampling	✓		✓			✓			✓	✓
16S rRNA Sequencing (Stool)	✓		✓			✓			✓	✓
Metagenomic Sequencing (Stool)	✓		✓			✓			✓	
SCFA Quantification (Stool)	✓		✓			✓			✓	
Prebiotic Metabolomics (Serum & Urine)									✓	
Untargeted Metabolomics (Serum)	✓		✓			✓			✓	
Olink Immune Profiling (Serum)	✓		✓			✓			✓	
PhophoFlow Immune Profiling (PBMCs)			✓						✓	
Blood Panel (Whole Blood)	✓		✓			✓			✓	✓
Dietary Assessment			✓						✓	
Physical Activity Assessment			✓						✓	
Wellbeing Questionnaires			✓						✓	
Cognitive Testing	✓		✓			✓			✓	✓

Blood and stool were collected at Weeks -2, 0, 3, 6 and 10. Unless otherwise noted, the "Baseline" measurement will be represented by the Week 0 time point. Specific analyses performed at each time point are listed above. Outcome analysis specified below will be conducted using data from two time points (baseline and end-of-intervention). Data from additional time points will be used in a discovery-based analysis not described in this document.

Primary Outcome: Cytokine Response Score

Time Frame: Baseline and 6 weeks

Change from baseline in Cytokine Response Score (CRS) at 6 weeks. The CRS is a single composite measure of cell-type specific activation of signaling pathways from ex vivo cytokine stimulation of blood samples. This provides a measure of immune response capacity which may be an indicator of immune fitness. The CRS will be calculated as described in Shen-Orr et al, Cell Systems, 2016. The CRS is the sum of 15 age-associated normalized cytokine responses identified in Shen-Orr et. al: In CD8+ T cells: IFNα pSTAT1, pSTAT3, pSTAT5; IL-6 pSTAT1, pSTAT3, pSTAT5; IFNγ pSTAT1; IL-21 pSTAT1; In CD_4+ T cells: IFNα pSTAT5; IL-6 pSTAT5; In B cells: IFNα pSTAT1; in monocytes: IL-10 pSTAT3; IFNγ pSTAT3; IFNα pSTAT3; IL-6 pSTAT3. Each feature is calculated as the fold change of the protein in the stimulated condition relative to its level in the unstimulated condition. That value is then normalized to the feature's range: normalized = (x - xmin)/xmax. The 15 normalized values are summed for the CRS.

Secondary Outcomes

Time Frame for each: Baseline and 6 weeks

1. **Microbiota composition**
    a. *16S sequencing*
    We will measure the change in alpha diversity from baseline to 6 weeks. We will be using the number of observed sequence variants ("species") determined by standard 16S rRNA amplicon sequencing (V3-V5 region followed by DADA2 to define error-corrected sequence variants) as our primary metric of alpha diversity. The units are the # of sequence variants.
    b. *Metagenomic sequencing*
    We will measure changes in functional attributes of the microbiota between baseline and 6 weeks. Shotgun metagenomic sequencing of stool samples enables the resolution of microbial genomes and their constituent genes. We will measure "reads per million reads" of each family of genes found in the microbiota to determine which bacterial functions are enriched and depleted over time.
2. **Untargeted Serum Metabolomics**
    We will use the high-throughput mass spectrometry pipeline developed by Han, et al. (2021) to identify metabolites found in serum. We will measure fold-change for each metabolite between baseline and six weeks.
3. **Microbiota function, SCFA production**
    We will measure change from baseline of short-chain fatty acids (SCFA) concentration at 6 weeks. We will measure acetate, propionate, butyrate, valerate and caproate, as well as pertinent isomers of each.
4. **Blood biomarker analysis**
    We will use the Olink proteomics platform to measure change in protein levels for a panel of 92 immune system-associated proteins for baseline and 6 weeks.
5. **Physical measurements**
    We will measure changes in the following anthropometrics from baseline to 6 weeks.
    a. Weight
    b. Waist Circumference

c. Blood pressure

6. **Blood panel analyses**
   We will measure changes in the following blood panel assays from baseline to 6 weeks.
   a. Total cholesterol
   b. HDL cholesterol
   c. Triglycerides
   d. Fasting glucose
   e. Fasting insulin

Additional Outcomes

1. **Targeted Serum and Urine Metabolomics**
   Metabolon, Inc. will provide quantification of prebiotic in serum and urine samples from the Week 6 time point only using targeted mass spectrometry.

Proposed statistical analyses

Blinding

This statistical analysis will be blinded. The analysis team will receive the participants labeled with "Group 1", "Group 2" or "Group 3" with no indication of the treatment associated with each group.

Statistical testing procedures

Correlating measurements with treatment

For each of the measurement modalities used in this study, we will frequently be comparing mean baseline-to-endpoint change across our three treatment groups (High Dose Prebiotic, Low Dose Prebiotic, Placebo). To do this, we will first average the values from the two baseline samples (Week -2 and Week 0). We will then use analysis of covariance (ANCOVA), with baseline measurement level as the covariate, which will test the null hypothesis that means are equal across all three groups. If any of these test results return a significant P-value, we will use Tukey's Honest Significant Differences to perform pairwise comparisons of each treatment arm in order to determine specifically which treatment arms differ significantly.

The following measurement modalities will use this approach:

- Cytokine response score
- Microbiota alpha diversity (via 16S sequencing)
- SCFA quantification
- Targeted serum and urine metabolomics
- Blood biomarker analysis (Olink)
- Physical measurements
- Blood panel analyses

We will utilize multiple hypothesis testing correction (Benjamini-Hochberg correction) within each measurement modality to protect against false positive findings. In addition, physical measurements and blood panel analyses will be subjected to standard confidence interval determination.

Microbiome function analysis (via metagenomics sequencing)

For analysis of microbiota function using metagenomics, we will first aggregate gene orthologs using the KEGG database and then determine the change in relative abundance of each gene family for each participant from baseline-to-endpoint. We will then identify KEGG functions and pathways whose change in abundance varies significantly by treatment arm using a negative binomial mixed model, using treatment arm as a fixed effect. P-values will be adjusted using the Benjamini-Hochberg method.

Correlating across measurement modalities

Using an approach derived from Wastyk, et al. (2020), the Spearman correlation between all parameters across measurement modalities will be calculated. For each measurement modality, participant-specific baseline-to-endpoint differences will be centered and scaled. Correlations will be filtered to only include host-microbe comparisons. Benjamini-Hochberg P-value correction will be used to control for Type I errors. If a participant has a missing sample for a given measurement modality, they will be removed from the analysis.

Impact of the COVID-19 pandemic

This study was in progress during the onset of the COVID-19 pandemic. This resulted in several individuals deviating from the standard treatment design. All participants will be included in the outcome analysis, but additional subset analyses will be conducted in an exploratory manner to better understand the impact of study design modification on the outcome variables. While we limited subject drop-out to only 2 individuals, there were other subjects who were unable to have specific samples collected at time points that depended on their enrollment date. As such, while our final sample size was 89 individuals who completed the intervention, there are specific analyses for which our sample size is less than 89, for example due to a participant missing a time point sample submission. In cases where participants are missing time point 0, week -2 will be substituted as a pre-intervention time point, if available. If a pre-intervention time point is not available or end of intervention is not available, that participant will be removed from the analysis for that data type. We expect sample drop-outs to be minimally disruptive for our planned analysis.

References

Shen-Orr, Shai S., et al. "Defective signaling in the JAK-STAT pathway tracks with chronic inflammation and cardiovascular risk in aging humans." Cell systems 3.4 (2016): 374-384.

Wastyk, Hannah C., et al. "Gut Microbiota-Targeted Diets Modulate Human Immune Status". bioRxiv (2020) 2020.09.30.321448.

Han, S., et al. "A metabolomics pipeline enables mechanistic interrogation of the gut microbiome". bioRxiv (2021) 2021.05.25.445684.