

EFFECT OF PROBIOTICS ON CARDIOVASCULAR BIOMARKERS

PRINCIPAL INVESTIGATOR:

Heather Zwickey, PhD (503) 552-1742

CO-INVESTIGATORS AND STAFF:

Jeremy Mikolai, ND (503) 552-1795

(Clinical Investigator)

Jennifer Ryan, ND (503) 552-1551 ext. 5047

(Project Coordinator/Student Researcher)

Morgan Schafer, MA (503) 552-1752

(Research Coordinator)

Mayen Dada, BA (503)-552-1744

(Research Coordinator)

Doug Hanes, PhD (503) 552-1741

(Biostatistician)

ABSTRACT

BACKGROUND & SIGNIFICANCE: Coronary artery disease is the leading cause of death in the United States. Individuals with higher levels of total cholesterol, LDL cholesterol, and triglycerides are at increased risk for coronary artery disease. In contrast, individuals with higher levels of HDL cholesterol have a decreased risk for coronary artery disease. Numerous animal and human studies have demonstrated that probiotics may have clinically desirable effects on cholesterol levels.

RESEARCH DESIGN & METHODS: For this pilot study, we are investigating the effect of probiotic *Saccharomyces boulardii* on cholesterol levels in twelve healthy individuals with high levels of total cholesterol and low levels of HDL cholesterol. All study participants will supplement their diets with 40 billion organisms per day of encapsulated *Saccharomyces boulardii* for 8 weeks. Outcome measures include HDL, LDL, total cholesterol, triglycerides, hs-CRP, fibrinogen, homocysteine, small dense LDL III, small dense LDL IV, total LDL particles, large buoyant HDL 2b, total HDL particles, non-HDL particles, VLDL particles, remnant lipoprotein (RLP), lipoprotein(a), apo B-100, and insulin levels measured at baseline, 4 weeks and 8 weeks.

A. SPECIFIC AIMS

Aim 1: The primary aim is to determine the effect of encapsulated *S. boulardii* on high density lipoprotein cholesterol (HDL). 20 billion organisms of *S. boulardii* will be administered twice per day for a total of 40 billion organisms per day (n=12). Outcomes will be collected at baseline, 4 weeks and 8 weeks.

- **Hypothesis 1:** 8 weeks of daily supplementation with 40 billion organisms of *S. boulardii* will increase blood levels of HDL.

Aim 2: Secondary aims include determining changes in low density lipoprotein cholesterol (LDL), total cholesterol, and triglycerides between baseline, 4 weeks and 8 weeks.

- **Hypothesis 2:** 8 weeks of daily supplementation with 40 billion organisms of *S. boulardii* will decrease blood levels of LDL, total cholesterol, and triglycerides.

Aim 3: Exploratory aims include determining changes in high-sensitivity C-reactive protein (hs-CRP), fibrinogen, homocysteine, small dense LDL III, small dense LDL IV, total LDL particles, large buoyant HDL 2b, total HDL particles, non-HDL particles, VLDL particles, remnant lipoprotein (RLP), lipoprotein(a), apo B-100, and insulin levels between baseline, 4 weeks and 8 weeks.

- **Hypothesis 3:** 8 weeks of daily supplementation with 40 billion organisms of *S. boulardii* will alter blood levels of hs-CRP, fibrinogen, homocysteine, small dense LDL III, small dense LDL IV, total LDL particles, large buoyant HDL 2b, total HDL particles, non-HDL particles, VLDL particles, remnant lipoprotein (RLP), lipoprotein(a), apo B-100, and insulin.

B. BACKGROUND and SIGNIFICANCE

B.1. Elevated Cholesterol and Coronary Artery Disease

Coronary artery disease is the leading cause of death in the United States. According to the American Heart Association, having high cholesterol levels in the blood is a major risk factor for coronary artery disease, which involves narrowing of the arteries that supply blood to the heart. Total cholesterol levels below 200 mg/dL are considered “desirable”; however, 99 million American adults have cholesterol levels that are at least 200 mg/dL and 34 million American adults have cholesterol levels that are at least 240 mg/dL, which is classified as “high”.^{1,2}

A typical blood cholesterol panel includes total cholesterol, low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL) and triglycerides. Individuals with higher levels of total cholesterol, LDL, and triglycerides are at increased risk for coronary artery disease. In contrast, individuals with higher levels of HDL, which is considered cardioprotective, have a decreased risk for coronary artery disease.² Interventions that raise HDL cholesterol have the potential to lower total cholesterol levels via a mechanism known as “reverse cholesterol transport”, in which cholesterol is transported from the peripheral tissues to the liver for removal from the body.³

In order to lower the risk of coronary artery disease, clinicians frequently use cholesterol-lowering medications in individuals with elevated cholesterol. According to a recent report from the Centers for Disease Control (CDC), 11% of the entire US population and 45% of the US population that is age 65 and over is on cholesterol-lowering medication.⁴ Statin drugs are the most frequently used class of cholesterol-lowering medication, yet up to 10% of patients on statin drugs experience adverse side effects including abdominal pain, diarrhea, constipation, flatulence, nausea, headache, fatigue, liver toxicity, muscle aches, soreness and weakness. Statins also interact with many other medications.^{2,5}

Considering that a significant percentage of patients using the most frequently prescribed treatments for high cholesterol experience adverse side effects, there is a need to validate additional interventions with fewer side effects. Probiotics are a promising therapy for the prevention and treatment of high cholesterol as well as coronary artery disease.

B.2. Probiotic Supplementation and Cholesterol Levels

Probiotics are live microorganisms that can be used clinically to prevent and treat numerous conditions, such as antibiotic-associated diarrhea, *Clostridium difficile*-associated diarrhea, irritable bowel

syndrome and atopic dermatitis. Examples of commonly used probiotics include bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* species, as well as yeast such as *Saccharomyces boulardii*.⁶ Probiotics have also been investigated as a treatment for high cholesterol. The effect of various probiotics on cholesterol levels has been examined in numerous human and animal studies.⁷⁻⁹

Researchers in Germany gave a group of volunteers yogurt that contained live cultures of *Lactobacillus acidophilus* and *Bifidobacterium longum*; they found that this intervention increased HDL.¹⁰ A group in Poland demonstrated an increase in HDL and a decrease in LDL in a group of volunteers who took *Lactobacillus plantarum*.¹¹ Researchers in Austria found that yogurt containing live cultures of the probiotic *Lactobacillus casei* raised HDL and lowered total as well as LDL cholesterol in a group of women.¹² Several other human participant studies have demonstrated that daily probiotic supplementation resulted in lowered LDL and total cholesterol levels.¹³⁻¹⁶

Animal studies have also demonstrated similar beneficial effects on lipid levels as human clinical trials.^{8,9,16,17} For example, Sindhu et al. gave a group of mice feed that contained the probiotics *Saccharomyces boulardii* and *Lactobacillus casei*. Compared to mice that had been given regular feed, the mice given feed that included probiotics had lower levels of LDL and total cholesterol, as well as higher levels of cardioprotective HDL.¹⁷ One of the probiotics used in this study, *S. boulardii*, has historically been used for complaints in the gastrointestinal system.¹⁸ To our knowledge, the effect of *S. boulardii* on cholesterol levels in human participants has not been tested previously.

Given that numerous animal and human studies have demonstrated that probiotics may affect cholesterol levels and that *S. boulardii* specifically has been shown to alter cholesterol levels in an animal model, this study aims to investigate the effect of *S. boulardii* on cholesterol levels in human participants.

B.3. Other Risk Factors for Coronary Artery Disease

Additional blood markers can be measured to determine the risk for the development of coronary artery disease. Cholesterol levels can be evaluated using techniques which separate lipids into lipoprotein subclasses known as lipoprotein particles.¹⁹ Similar to cholesterol levels, elevations in certain lipoprotein particles are associated with increased risk for cardiovascular disease, while others are considered cardioprotective. For example, small dense LDL associated with increased risk for coronary artery disease, while large-buoyant HDL 2b is associated with the metabolism and removal of cholesterol from the body.^{20,21} Examples of other lipoprotein particle measurements include total LDL particles, total HDL particles, non-HDL particles, VLDL particles, remnant lipoprotein (RLP), lipoprotein(a), and apo B-100.¹⁹

Elevated levels of inflammatory markers are also considered risk factors for the development of coronary artery disease. Examples of inflammatory markers include high sensitivity C-reactive protein (hs-CRP), fibrinogen, and homocysteine. The higher the levels of these markers in the blood, the higher the likelihood of a coronary event such as a heart attack or a stroke.²

Impaired blood sugar regulation may also predispose an individual to the development of cardiovascular disease.² Elevated levels of fasting insulin are correlated with insulin resistance, which is associated with a higher risk of stroke.²²

In addition to measuring HDL, LDL, total cholesterol and triglycerides, this study will examine the effect of *Saccharomyces boulardii* on the levels of small dense LDL III, small dense LDL IV, total LDL particles, large buoyant HDL 2b, total HDL particles, non-HDL particles, VLDL particles, remnant lipoprotein (RLP), lipoprotein(a), apo B-100, hs-CRP, fibrinogen, homocysteine, and insulin. These additional outcomes are exploratory, as to our knowledge there have been no prior animal or human studies that have measured these biomarkers with the use of *S. boulardii*. However, monitoring these additional biomarkers may elucidate mechanisms involved in the alteration of cholesterol by probiotics.

B.4. *S. boulardii* Dosage

A systematic review and meta-analysis of *S. boulardii* published in 2010 examined the effectiveness of *S. boulardii* for acute and chronic gastrointestinal disease applications. The 27 studies included in the review encompassed over 5000 adult participants and no major adverse events were reported. *S. boulardii* was given safely at dosages ranging from 2 to 60 billion organisms per day for durations ranging from 1 to 26 weeks.¹⁸

A Cochrane Review published in 2011 examined the effectiveness of probiotics for the prevention of antibiotic-associated diarrhea in children. The authors concluded that dosages of *S. boulardii* as high as 40 billion organisms per day may be necessary to be therapeutic.²³

Although this current study will examine the effect of *S. boulardii* on cholesterol in adults, these previous reviews are a good indication of the dosing that may be necessary to demonstrate changes in cholesterol and other cardiovascular biomarkers. Taking these previous studies and reviews into consideration, we have selected a dosage of 40 billion organisms per day and a duration of 8 weeks for this pilot study. Future studies could involve testing the effect of *S. boulardii* on cardiovascular biomarkers at varying dosages.

B.5. Cytokines

Cytokines are molecules involved in intercellular communication and inflammation, including cardiovascular inflammation. Levels of specific cytokines have been correlated with lipid levels as well as cardiovascular inflammatory markers.^{24,25}

Probiotics, including *S. boulardii*, have been shown to alter levels of cytokines.^{11,18} Establishing a correlation between probiotic supplementation, levels of lipids and cardiovascular inflammatory markers, as well as levels of cytokines may give a more complete picture of the mechanisms involved.

In addition to analyzing blood samples to measure the specific aims in this study, a small portion of the blood samples collected will be stored for possible secondary cytokine analysis in a subsequent investigation. If the results of the currently proposed study warrant analysis of these samples for cytokine levels, a PRAF or a separate IRB application will be submitted.

C. PRELIMINARY DATA

In order to facilitate the design of this study, one of the study investigators opted to collect pre-pilot data on a volunteer basis. Over a period of 8 weeks, the volunteer took 20 billion organisms of *S. boulardii* twice per day for a total of 40 billion organisms per day. The *S. boulardii* was purchased from Thorne Research (Dover, ID, USA). HDL, total cholesterol, LDL, triglycerides, hs-CRP, fibrinogen, homocysteine, small dense LDL III, small dense LDL IV, total LDL particles, large buoyant HDL 2b, total HDL particles, non-HDL particles, VLDL particles, remnant lipoprotein (RLP), lipoprotein(a), apo B-100, and insulin were measured at baseline, 4 weeks, and 8 weeks. Serum samples were sent to SpectraCell Laboratories (Houston, TX) and Quest Diagnostics (San Juan Capistrano, CA) for analysis. No adverse effects or events were noted.

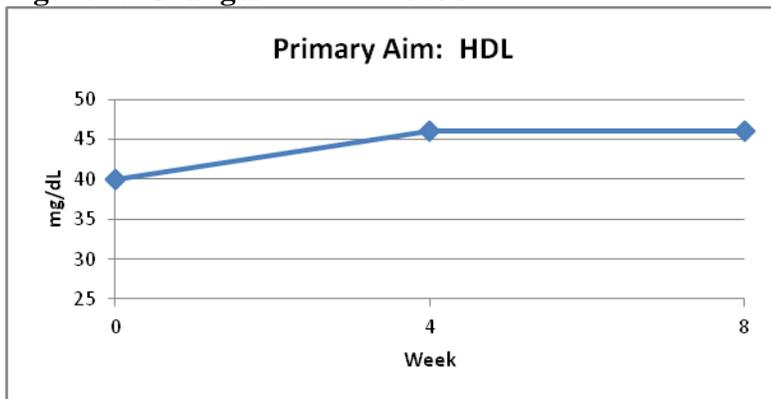
C.1. Primary Aim

Changes in HDL, the primary aim, are shown in Table 1 and Figure 1. After 4 weeks of daily supplementation with *S. boulardii*, HDL cholesterol increased by 15%; this increase was maintained when levels were measured again at 8 weeks.

Table 1: Primary Aim

Outcome Measure	Reference Range	Baseline	After 4 Weeks	After 8 Weeks	% Change Baseline to 8 Weeks
HDL	> 40 (mg/dL)	40	46	46	↑ 15%

Figure 1: Changes in HDL Levels



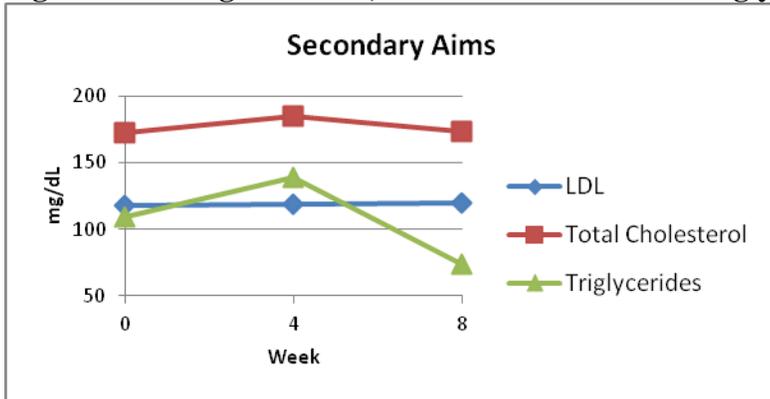
C.2. Secondary Aims

Changes in secondary aims are shown in Table 2 and Figure 2.

Table 2: Secondary Aims

Outcome Measure	Reference Range	Baseline	After 4 Weeks	After 8 Weeks	% Change Baseline to 8 Weeks
LDL	40-130 (mg/dL)	118	119	120	↑ 1.7%
Total Cholesterol	< 200 (mg/dL)	172	185	173	↑ 0.6%
Triglycerides	30-150 (mg/dL)	109	139	74	↓ 32%

Figure 2: Changes in LDL, Total Cholesterol and Triglycerides



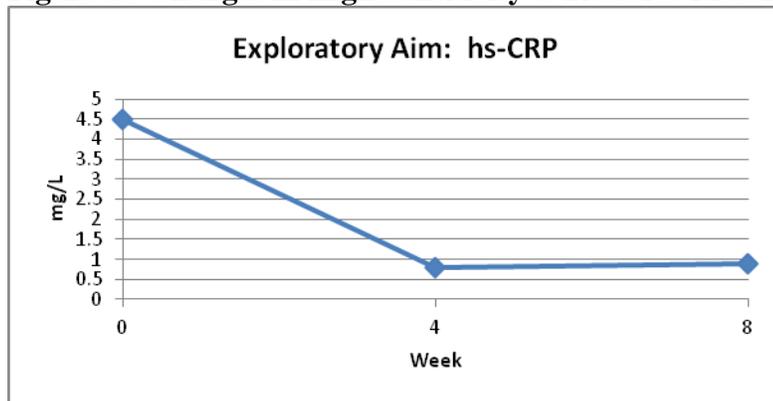
C.3. Exploratory Aims

Changes in exploratory aims are shown in Table 3 and Figure 3.

Table 3: Exploratory Aims

Outcome Measure	Reference Range	Baseline	After 4 Weeks	After 8 Weeks	% Change Baseline to 8 Weeks
hs-CRP	< 3.0 (mg/L)	4.5	0.8	0.9	↓ 80%
Fibrinogen	175-425 (mg/dL)	377	312	305	↓ 19%
Homocysteine	< 11.0 (umol/L)	6.8	7.1	6.8	0%
Small Dense LDL III	< 300 (nmol/L)	380	312	277	↓ 27%
Small Dense LDL IV	<100 (nmol/L)	64	63	69	↑ 8%
Total LDL Particles	< 900 (nmol/L)	818	816	874	↑ 7%
Large-Buoyant HDL 2b	> 1500 (nmol/L)	1399	1560	1828	↑ 31%
Total HDL Particles	> 7000 (nmol/L)	7618	7342	7397	↓ 3%
Non-HDL Particle Numbers	< 1000	885	898	918	↑ 4%
VLDL Particles	< 85 (nmol/L)	67	82	44	↓ 34%
Remnant Lipoprotein (RLP)	< 150 (nmol/L)	94	104	106	↑ 13%
Lipoprotein(a)	6.0-29.9 (mg/dL)	17.8	16.7	15.5	↓ 13%
Apo B-100	40-100 (mg/dL)	85	86	81	↓ 5%
Insulin	4.0-35.5 (uIU/mL)	9.2	8.8	5.1	↓ 45%

Figure 3: Changes in High Sensitivity C-Reactive Protein (hs-CRP)



High sensitivity C-reactive protein levels decreased by 80% after 4 weeks and the decrease was maintained at the 8 week measurement. Many other exploratory outcomes were altered, including some that were altered by more than 20%: small dense LDL III, large buoyant HDL 2b, VLDL particles, and insulin.

This preliminary data suggests that *S. boulardii* may have clinically desirable effects on lipid levels and other risk factors for coronary artery disease. A pilot study with a larger sample size is necessary to expand upon these findings.

D. RESEARCH DESIGN AND METHODS

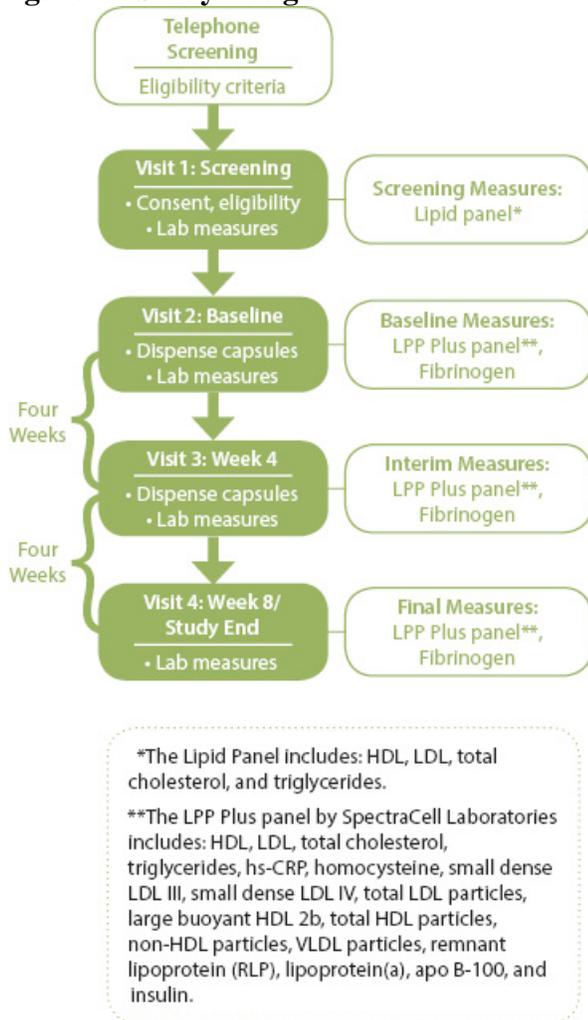
D.1. Study Design Overview

The purpose of this pre-post pilot study is to evaluate the effect of *Saccharomyces boulardii* on cholesterol levels and cardiovascular biomarkers. Twelve generally healthy participants will be enrolled in the trial. All enrolled participants will take encapsulated *Saccharomyces boulardii* daily for 8 weeks.

The study consists of a telephone screening, one screening visit and three study visits. Following a telephone screening, potential participants will be consented and their blood will be drawn at the screening visit to determine if they meet additional inclusion criteria. If they qualify, they will be invited back to enroll in the study and begin taking 4 capsules of *Saccharomyces boulardii* twice per day for 8 weeks. Additional blood will be drawn at baseline, at 4 weeks and at 8 weeks. (See Figure 4.)

Outcome measures include HDL, LDL, total cholesterol, triglycerides, hs-CRP, fibrinogen, homocysteine, small dense LDL III, small dense LDL IV, total LDL particles, large buoyant HDL 2b, total HDL particles, non-HDL particles, VLDL particles, remnant lipoprotein (RLP), lipoprotein(a), apo B-100, and insulin levels measured at baseline, 4 weeks and 8 weeks.

Figure 4: Study Design



D.2. Study Population

The target population for this study is otherwise healthy participants between 18-65 years of age, who have elevated levels of total cholesterol with low levels of HDL cholesterol. For this study, elevated cholesterol will be defined as ≥ 200 mg/dL and low HDL will be defined as < 50 mg/dL. Participants will also be chosen based on additional inclusion and exclusion criteria described below. These criteria were chosen to establish general health of the participants and minimize factors that may interfere with the intervention.

D.3. Inclusion Criteria

- Age 18-65.
- Total Cholesterol ≥ 200 mg/dL
- HDL Cholesterol ≤ 50 mg/dL
- Willingness to fast for 12 hours before blood draws and to abstain from alcoholic beverages for 24 hours before blood draws.
- Willing to provide informed consent.
- Willing and able to complete all aspects of the trial.

D.4. Exclusion Criteria

- Systolic blood pressure ≥ 160 mmHg or a diastolic blood pressure ≥ 100 mmHg upon screening
- Body mass index (BMI) > 45
- Currently taking a probiotic supplement or took a probiotic supplement within the last month.
- Currently taking an oral antifungal medication or took an antifungal medication within the last month.
- Currently taking any of the following medications or supplements (or they were taken within the last 2 months):
 - Cholesterol lowering prescription medications.
 - Supplements that may lower cholesterol (e.g. Niacin, Guggul, Red Rice Yeast, Garlic (encapsulated), Phytosterols, Polycosanols, Fish oil, and Coenzyme Q10).
 - Immunosuppressant medications.
 - Corticosteroid medications.
 - Other medications and supplements to be evaluated by the investigators on a case-by-case basis.
- Planning to make significant dietary changes during the study period.
- Planning to start a new exercise regime during the study.
- Current or past history of any of the following:
 - Heart disease, including but not limited to: Myocardial infarction, Stroke, Congestive heart failure (CHF), Valvular disease, and Rheumatic fever.
 - Heart surgery, including but not limited to: Coronary angioplasty, Stent placement, Coronary artery bypass surgery, valve repair or replacement
 - (Participants who had congenital heart defects that were repaired before the age of 1 will not be excluded unless they have prosthetic valves)
 - Cardiac arrhythmia including have a pacemaker
 - Abnormal EKG
 - Abnormal echocardiogram
 - Previously diagnosed diabetes
 - Previously diagnosed immunodeficiency disorder (e.g. HIV, AIDS).

- Previously diagnosed bowel disease (e.g. Crohn’s Disease, Ulcerative colitis, Irritable Bowel Syndrome, Diverticulitis, Celiac Disease).
- Previously diagnosed liver disease (including fatty liver disease).
- Previously diagnosed kidney disease.
- Previously diagnosed hypothyroidism that is untreated.
- Cancer within the last 5 years.
- Family history of premature CAD, defined as the following:
 - Myocardial infarction or sudden death before the age of 55 in their father or other male first-degree relative (brother or son)
 - Myocardial infarction or sudden death before the age of 65 in their mother or other female first-degree relative (sister or daughter)
- Currently have a central venous catheter in place.
- Pregnant or lactating women.
- Women who plan to become pregnant within the next six months.
- Difficulty or aversion to swallowing capsules/pills.
- Known intolerance or allergy to probiotics or rice.
- Participants may also be excluded at screening or baseline if their blood work reveals clinical alert values. (See section E.5.1. for additional details.)

D.5. Screening and Study Activities

D.5.1. Telephone Screening

Potential participants will be screened over the phone by study personnel prior to coming in for the first visit. A standardized telephone script will be used. The telephone screening will take approximately 10-15 minutes. The consent form will be mailed to the participant prior to Visit 1; they will be instructed to review the form ahead of time but not to sign it until Visit 1. Participants will be informed that they will be asked to fast for 12 hours and abstain from alcoholic beverages 24 hours before each of the four blood draws

D.5.2. Visit 1: Screening

All screening and study visits will take place at the Helfgott Research Institute. Potential participants that meet eligibility criteria over the phone will be scheduled for a screening visit. At the screening visit, participants will provide informed consent and complete an eligibility and health history questionnaire. We will measure their height, weight, and blood pressure. Body mass index (BMI) will be calculated using the height and weight measurements and participants may be excluded if either their BMI or blood pressure reading exceeds the values specified in the exclusion criteria. A fasting blood sample will be taken to determine if they are eligible for the study based upon additional inclusion criteria. Approximately half a tablespoon (8 mL) of blood will be collected. The visit is expected to take 45-60 minutes.

Following Visit 1, 10-Year Framingham risk score will be calculated according to guidelines from the National Cholesterol Education Program (NCEP).² The calculation will be based on the following information from each participant: gender, age, total and HDL cholesterol (upon screening), smoking status, systolic blood pressure (upon screening), and whether or not they are taking blood pressure medication. Participants with a 10-Year Framingham risk score $\geq 20\%$ will be excluded from the study.

Following Visit 1 and subsequent Framingham risk score calculation, participants will be contacted by phone and told whether they qualify for the study or not. Participants meeting all final eligibility criteria will be invited to participate in the intervention phase of the study and they will be scheduled for Visit 2.

D.5.3. Visit 2: Baseline

At Visit 2, participants will be provided with encapsulated *S. boulardii*, instructions on how to take the capsules, and a Capsule Intake Log to track the capsules that they take. Participants will also have a fasting sample of their blood drawn, as a measurement of their baseline levels of all outcome measures. Study personnel will answer participant questions and confirm the date and time of Visit 3. This visit is expected to take 30-45 minutes.

Two weeks after Visit 2, study personnel will contact participants to answer questions, and to assess for adverse events and compliance.

D.5.4. Visit 3: 4 Weeks

Visit 3 will take place 4 weeks after Visit 2. At Visit 3, participants will be assessed for adverse events, fill out a mid-study questionnaire, and will have a fasting sample of their blood drawn. They will return their first Capsule Intake Log and their *S. boulardii* bottles with any unused capsules. The first Capsule Intake Log will be reviewed with each participant to determine if there were any problems with taking the capsules. Participants will be provided with additional bottles of *S. boulardii* and a second Capsule Intake Log to continue tracking the capsules that they take. Study personnel will answer participant questions and confirm the date and time of Visit 4. This visit is expected to take 30-45 minutes.

Two weeks after Visit 3, study personnel will contact participants to answer questions, monitor for adverse events and compliance.

D.5.5. Visit 4: 8 Weeks/Study End

Visit 4 will take place 4 weeks after Visit 3. At Visit 4, participants will fill out a study completion questionnaire, be assessed for adverse events, and will have a fasting sample of their blood drawn. Their height, weight and blood pressure will also be measured. Participants will return Capsule Intake Log 2 and their *S. boulardii* capsule bottles with any unused capsules. Capsule Intake Log 2 will be reviewed with each participant to determine if there were any problems with taking the capsules. The visit is expected to take 45-60 minutes.

Table 4: Schedule of Measurements and Data Collection

	Telephone Screening	Visit 1 Screening	Visit 2 Baseline	Visit 3 Week 4	Visit 4 Week 8
Telephone Screening	•				
Inclusion/Exclusion	•	•			
Informed Consent		•			
Eligibility Questionnaire		•			
Height, Weight, Blood Pressure		•			•
HDL Cholesterol		•	•	•	•
LDL Cholesterol		•	•	•	•
Total Cholesterol		•	•	•	•
Triglycerides		•	•	•	•
hs-CRP			•	•	•
Fibrinogen			•	•	•
Homocysteine			•	•	•
Small Dense LDL III			•	•	•
Small Dense LDL IV			•	•	•
Total LDL Particles			•	•	•
Large-Buoyant HDL 2b			•	•	•
Total HDL Particles			•	•	•
Non-HDL Particle Numbers			•	•	•
VLDL Particles			•	•	•
Remnant Lipoprotein (RLP)			•	•	•
Lipoprotein(a)			•	•	•
Apo B-100			•	•	•
Insulin			•	•	•
Dispense Capsules			•	•	
Capsule Intake Log (1)			Given	Returned	
Capsule Intake Log (2)				Given	Returned
Capsule Count				•	•
Mid-Study Questionnaire				•	
Assess for Adverse Events*				•	•
Completion Questionnaire					•

*Adverse events will also be assessed by phone during the 8 week study period.

D.6. Compliance

Participant compliance will be monitored by reported capsule intake as recorded by participants on the capsule intake logs and by a capsule count completed by study personnel when the bottles and unused capsules are returned at Visit 3 and Visit 4.

D.7. Blood Collection and Analysis

Blood collection at Visit 1 will consist of a venous blood draw into one serum separator tube. Approximately 4ml of blood will be drawn from each participant. This sample will be transported to the NCNM Clinic Laboratory for analysis on the same day the sample is collected. After analysis, blood samples will be destroyed as per the laboratory standards of the NCNM Clinic Laboratory.

Blood collection at Visits 2, 3 and 4 will consist of a venous blood draw into one serum separator tube and one sodium citrate tube. Approximately 8ml of blood will be drawn from each participant. Blood collected in the serum separator tube will be sent to SpectraCell Laboratories for analysis; these samples will be picked up by FedEx on the same day the sample is collected. Blood collected in the sodium citrate tube will sent to Quest Diagnostics for analysis; these samples will be picked up by local courier on the same day the sample is collected. After analysis, blood samples will be destroyed as per the laboratory standards of SpectraCell Laboratories and Quest Diagnostics.

Test results from the NCNM Clinic will be sent to a secure fax machine. Test results from SpectraCell Laboratories and Quest Diagnostics will be accessed via secure electronic delivery systems.

In addition, a small portion of the blood collected at Visits 2, 3, and 4 will be frozen and stored for possible secondary cytokine analysis in a subsequent investigation. These samples will be de-identified and stored for a period of up to two years after the participant has completed the study. If the results of the currently proposed study warrant analysis of these stored samples for cytokine levels, a PRAF or a separate IRB application will be submitted.

D.8. Statistical and Methodological Considerations

D.8.1. Randomization and Blinding

Since this is a pilot study, all enrolled participants will be given the same intervention and therefore will not be randomized or blinded.

D.8.2 Statistical Analysis

For Specific Aim 1, data will be analyzed by submitting baseline, 4-week, and 8-week HDL values to a one-way repeated measures ANOVA. Repeated measures analysis will test for significant within-participants changes in HDL levels over time. If the global effect of timepoint in the ANOVA is significant, then we will apply Tukey Posthoc comparisons in order to test for significant changes in the 0-4, 4-8, and 0-8 week time intervals. This will be important in establishing experimental timelines for future investigations.

The above analyses will be performed assuming that the variables are normally distributed. If a test of normality proves otherwise, any non-normal variables will be analyzed using a non-parametric, ranked ANOVA model. Since non-parametric tests are generally weaker than their parametric analogues, the non-parametric test will serve as a more conservative, confirmatory analysis of results obtained with the original ANOVA.

For Specific Aim 2, these same analyses will be repeated for measurements of LDL, total cholesterol, and triglycerides. For Specific Aim 3, these same analyses will be repeated for hs-CRP, fibrinogen, homocysteine, small dense LDL III, small dense LDL IV, total LDL particles, large buoyant HDL 2b,

total HDL particles, non-HDL particles, VLDL particles, remnant lipoprotein (RLP), lipoprotein(a), apo B-100, and insulin.

D.8.3 Intention to Treat Analysis and Missing Data

We plan to conduct an intention to treat analysis, as is appropriate for tests of clinical interventions. The defining feature of intention to treat analysis is that all available data are included regardless of participant compliance. Although every effort will be made to ensure complete data, it is anticipated that some data will be missing due to dropouts, which would result in missing follow-up measurements for these participants.

Intention to treat analysis can and will be implemented in two valid ways. Each approach has unavoidable shortcomings and strengths, but together they will provide a much clearer picture of the efficacy of the treatment protocol.

First, we will exclude from the analysis participants who do not complete testing at both baseline and 4 weeks, but retain all data for participants who dropped out after the fourth week. We will include all such participants, whether or not they are deemed compliant with the treatment protocol. This will allow us to test for changes in HDL among participants for whom measurements could be compared over time. However, one criticism of this approach is that there may be systematic differences between drop-outs and completers, introducing a bias in the results.

The second approach is more conservative and will serve as a check on results obtained in the first analysis. For participants who complete baseline, but not 4 weeks and 8 weeks, we will use the last-observation-carried-forward (LOCF) approach to missing data to impute 4 week and 8 week values. In other words, missing values for measurements at the fourth and eighth week will be entered as equal to the same measurement obtained at baseline. We will also use this method to impute 8 week values for participants who complete baseline and 4 weeks, but not 8 weeks. This may weaken overall results and it is a questionable practice to impute data because it remains unknown what actually happened with the participant. This approach will ultimately reduce the power of the study by adding variability and decreasing the difference in pretest-posttest means, but this analysis will account for effects that might have differed between drop-outs and completing participants.

D.8.4. Power Calculation and Sample Size

To calculate power for a single paired-samples comparison over one time period, we used a previously obtained value for standard deviation in HDL measurement (13.92 mg/dL) and effect of treatment on mean HDL (13.15 mg/dL), using an alternative, but related, treatment.¹⁰ With these values, we calculate a power of 76% with 10 of the 12 enrolled participants completing the study. Although it would be desirable to have more participants, the large effects demonstrated in the Kiessling study indicate a good chance of success, even with the small sample size available for this pilot study. In the event that we obtain non-significant trends, we will conduct post-hoc analysis to estimate the size of sample that may be sufficient to yield significance in subsequent research.

D.9. Work Plan

The intervention is expected to begin upon approval from the IRB and continue for the next 8 months. Data entry, quality control and preparation, and participant management will be ongoing throughout the study. The principal investigator will oversee the management of the participants: intervention management, and analysis, which will be carried out by the research coordinators, clinical investigator, laboratory coordinator, research statistician, and student researcher. Upon completion of the study, the data will be analyzed and used to develop further trials.

D.10. Study Timeline

This study is planned to be completed within twelve months of IRB approval.

Table 5: Study Timeline

Activity	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
Finalize Protocol and Submit to IRB	•											
Address IRB Required Revisions (as Needed)		•										
Coordinate Supplement Shipping		•										
Recruitment			•	•	•	•	•	•				
Screening and Study Visits			•	•	•	•	•	•	•	•		
Data Summary and Analyses											•	•
Reports, Manuscripts											•	•

E. HUMAN SUBJECTS RESEARCH

E.1. Characteristics of the Study Population

Up to 12 participants will be enrolled in order to have at least 10 complete the trial. Participants will be chosen based on the inclusion and exclusion criteria that were defined in detail in the research design and methods section above. Many of these criteria were chosen to establish general health of the participants and to exclude participants who may have contraindications to the intervention. Contraindications to the intervention will be discussed in detail in the potential risks and procedures to minimize risks sections below.

E.2. Recruitment

Participants will be recruited from the general population, as well as NCNM faculty, staff, students, and the NCNM clinics. Advertisements will be posted on the school and clinic bulletin boards and on bulletin boards at other local schools, apartment complexes and businesses. Electronic communications and media postings will be used, including Craigslist and the NCNM web page. We may also contact individuals recruited in the past for previous studies, who indicated that they would like to be contacted about future study opportunities.

Appropriate language will be included in the consent form to avoid coercion. It will be emphasized that participation in the study is completely voluntary. Copies of all advertisements will be submitted for IRB approval.

E.3. Informed Consent

Each participant will sign an approved written informed consent form during the screening visit. The consent form will detail the purpose of the study, the requirements for participation, and the potential benefits and risks. It will also document that participation is voluntary, may be terminated by the participant at any time, and will not affect the ability to participate in future research studies. Participants will be given a signed copy of the consent form for their records.

E.4. Sources of Research Material

E.4.1. Data Collection Methods

The procedures for obtaining research material will include a telephone screening form, medical history form, and blood collection. Data will be collected directly from study participants specifically for research purposes. All blood collection will be performed at the Helfgott Research Institute. All data used for this project will be obtained only after receiving participant informed consent.

E.4.2. Confidentiality and Data Storage

Confidentiality: All participant information, and even the fact that an individual is in the study, is considered confidential. Confidentiality will be assured in this study through several mechanisms. During interviews and treatments, the investigators and study coordinator will ensure physical privacy by conducting interviews in a closed room. All data are stored in a secured area accessible only to study staff. After enrollment, each participant will be assigned an anonymous study ID number, which will then be used on all study forms. All study forms, and paper records that contain participant information (e.g., address lists and phone lists) will be kept in secured, locked areas when not in use. In addition, such materials, when in use, will be kept away from public scrutiny. Access to all participant data and information, will be restricted to authorized personnel. In the case of computerized information, access to the study data on computers will be password protected. Staff members receive individualized account numbers and passwords that allow them access only to those elements of the data management system to which they are authorized. When the study database is made available to the project office, it will not include actual identities or contact information for participants. Finally, participants will not be identified by name in any reports or publications, nor will data presented in such a way that the identity of individual participants can be inferred. All staff are trained and annually re-certified regarding these procedures.

E.4.3. Data Integrity

Participant's completed forms will be reviewed for completeness and consistency by the study coordinator. Data maintained at NCM will be backed up daily and archived off-line. Data entry will be completed by study staff. Duplicate data entry will be performed to ensure data integrity. The project coordinator, principal investigator, clinical investigator, study statistician, and research assistants will meet as needed to review data and adjudicate any issues identified.

E.5. Potential Risks and Procedures for Protecting Individuals from Risk

This study should not pose any major health risks to participants. The study protocol includes features to minimize potential risks. All study visits and procedures will be conducted by qualified study personnel.

E.5.1. Establishing General Health of the Participants

Only generally healthy individuals between the ages of 18 and 65 will be eligible to participate in the study. Potential harm will be minimized by excluding participants with the conditions detailed in the methods section under exclusion criteria.

We will also monitor for clinical alert values at screening, baseline, and throughout the study. Clinical alert values may represent a pathological state at such variance to normal to be considered potentially life threatening. If a participant or potential participant is found to have a clinical alert value on a blood test, they will be contacted by the clinical investigator, Dr. Jeremy Mikolai, and may be excluded or removed from the study. If an already enrolled participant wishes to continue in the study, the potential risks addressed in the consent form will be discussed again with the participant.

Clinical alert values are specified below in Table 6. The clinical alert value for total cholesterol is defined as ≥ 275 mg/dL for the purpose of this study only. (Note that not all outcome measures have defined clinical alert values.)

Table 6: Clinical Alert Values

Outcome Measure	Clinical Alert Value
HDL Cholesterol	<25 mg/dL ²⁶
LDL Cholesterol	≥ 190 mg/dL ²⁶
Total Cholesterol	>275 mg/dL
Triglycerides	> 500 mg/dL ²⁶
hs-CRP	> 10 mg/L ²⁶
Fibrinogen	<100 mg/dL ²⁶

In addition, investigators will follow this clinical alert protocol following week 4 (Visit 3) under the following circumstances:

- HDL decreases by $\geq 30\%$ from baseline (Visit 2)
- LDL cholesterol, total cholesterol, hs-CRP and/or fibrinogen increases by $\geq 30\%$ from baseline (Visit 2)

E.5.2. Risks Associated with Blood Draws

Participants may experience slight discomfort during these procedures, but every effort will be made to ensure that participants are as comfortable as possible. The main risks associated with drawing blood are infection, bruising, redness, discomfort, or bleeding at the needle puncture site. Occasionally, individuals may feel lightheaded or faint during or immediately after a blood sample is collected. There is also a rare risk of swelling around the vein. Following the blood collection, participants may experience slight bruising and pain at the collection site.

To minimize risk, blood collection will be conducted by licensed phlebotomists and naturopathic physicians using aseptic technique.

E.5.3. Risks Associated with *Saccharomyces boulardii*

Saccharomyces boulardii is a probiotic supplement that has been used since the 1950s and has been investigated worldwide. It has been studied extensively as a treatment in numerous acute and chronic gastrointestinal illnesses. A review article published in 2010 noted that *Saccharomyces boulardii* had been given safely to over 5000 participants in the 27 randomized controlled trials that were included in the review.¹⁸

Rare cases of fungemia have been reported in seriously ill patients with central venous catheters.²⁷ To minimize risk in this study, only generally healthy participants will be included and we will specifically exclude individuals who have a central venous catheter. We will also exclude individuals who have a known immunodeficiency disorder and individuals currently taking immunosuppressant medications. Note however that there were no cases of fungemia reported in the McFarland review noted above.¹⁸

According to the supplier, Pure Encapsulations, probiotics may result in mild flatulence. It is not believed that the planned supplementation will have any major adverse effects, but it is possible for anyone taking an oral supplement to experience gastrointestinal discomfort such as gas, bloating or digestive upset. We will monitor symptoms and any potential adverse reactions by asking participants to record any unusual symptoms on their capsule intake logs. These logs will be reviewed with participants at study visits and participants will also be contacted by phone between study visits to monitor for adverse reactions.

In addition, participants will be given the option of contacting the clinical investigator, Dr. Jeremy Mikolai, who will record all events in the participant's file and an adverse event log. He will triage the participant on the phone to determine if immediate emergency care is needed, otherwise an office visit within one day will be scheduled to evaluate and treat any adverse reactions. The clinical investigator will initially determine severity of adverse events and appropriate measures will be taken to ensure safety of all study participants if an event has occurred. The IRB will be notified of adverse events and unanticipated problems according to established policies.

If an adverse event has occurred during the study period, participants will be followed up with on an as needed basis after their participation in the study has concluded.

E.5.4. Risks Associated with a Breach of Confidentiality.

There is a small risk that information about a study participant could be inadvertently disclosed to non-study personnel. Procedures to minimize this risk have been described in section E.4.2. (Confidentiality and Data Storage).

E.6. Potential Benefits

Potential benefits to participants include access to laboratory results. All screened participants will receive copies of their screening laboratory results. If they are enrolled in the study, they will also receive copies of their laboratory results that were collected at week 8 of the study. There may be no other direct benefits to the participants other than the personal satisfaction of being part of a study that may further scientific knowledge in the prevention of cardiovascular disease.

E.7. Incentives

Financial incentives will not be offered to participants.

F. IMPORTANCE OF KNOWLEDGE GAINED

Coronary artery disease is the leading cause of death in the United States. In order to lower the risk of coronary artery disease, clinicians frequently use cholesterol-lowering medications in individuals with elevated cholesterol. However, a significant percentage of patients using cholesterol-lowering medications experience adverse side effects and there is a need to validate additional interventions with fewer side effects. Probiotics are a promising therapy for the prevention and treatment of high cholesterol as well as coronary artery disease.

Additionally, based on the knowledge to be gained from this study we may decide to apply for funding for a larger study.

G. CITED REFERENCES

1. Roger VL et al. Heart disease and stroke statistics – 2011 update: a report from the American heart association. *Circulation*. 2011 Feb 1;123(4):e18-e209.
2. Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report. *Circulation*. 2002 Dec 17;106(25):3143-421.
3. Tall AR. An overview of reverse cholesterol transport. *Eur Heart J*. 1998;19(suppl A):A31-A35.
4. National Center for Health Statistics. Health, United States, 2010: With Special Feature on Death and Dying. Hyattsville, MD. 2011.
5. Hou R & A Goldberg. (2009). “Lowering low-density lipoprotein cholesterol: statins, ezetimibe, bile acid sequestrants, and combinations: comparative efficacy and safety.” *Endocrinol Metab Clin N Am* 38(1): 79-97.
6. Vanderfoof J & R Young. (2008.) “Probiotics in the United States.” *Clin Infect Dis* 46(Suppl 2): S67-72.
7. de Roos N, Katan M. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. *Am J Clin Nutr*. 2000 Feb;71(2):405-11.
8. Lye H et al. (2009.) “The improvement of hypertension by probiotics: effects on cholesterol, diabetes, rennin, and phytoestrogens.” *Int J Mol Sci* 10(9): 3755-3775.
9. Ooi L & M Liong. (2010.) “Cholesterol-lowering effects of probiotics and prebiotics: a review of in vivo and in vitro findings.” *Int J Mol Sci* 11(6): 2499-2522.
10. Kiessling G et al. Long-term consumption of fermented dairy products over 6 months increases HDL cholesterol. *Eur J Clin Nutr*. 2002 Sep;56(9):843-9.
11. Naruszewicz M et al. Effect of *Lactobacillus plantarum* 299v on cardiovascular disease risk factors in smokers. *Am J Clin Nutr*. 2002 Dec;76(6):1249-55.
12. Fabian E, Elmadfa I. Influence of daily consumption of probiotic and conventional yoghurt on the plasma lipid profile in young healthy women. *Ann Nutr Metab*. 2006;50(4):387-93.
13. Hlivak P et al. (2005). "One-year application of probiotic strain *Enterococcus faecium* M-74 decreases serum cholesterol levels." *Bratisl Lek Listy* 106(2): 67-72.
14. Larkin TA et al. Dietary combination of soy with a probiotic or prebiotic food significantly reduces total and LDL cholesterol in mildly hypercholesterolaemic subjects. *Eur J Clin Nutr*. 2009 Feb;63(2):238-45.
15. Schaafsma G et al. (1998.) “Effects of milk product, fermented by *Lactobacillus acidophilus* and with fructo-oligosaccharides added, on blood lipids in male volunteers.” *Eur J Clin Nutr* 52(6): 436-440.
16. Xiao JZ et al. Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult volunteers. *J Dairy Sci*. 2003 Jul;86(7):2452-2461.

17. Sindu S, Khetarpaul N. Effect of feeding probiotic fermented indigenous food mixture on serum cholesterol levels in mice. *Nutr Res.* 2003 Aug;23(8):1071-1080.
18. McFarland, LV. Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients. *World J Gastroenterol.* 2010 May 14;16(18):2202-22.
19. Handbook of Lipoprotein Testing, 2nd Edition, American Association for Clinical Chemistry, Inc. Press, Washington DC, 2000.
20. Gardner CD et al. Association of small low-density lipoprotein particles with the incidence of coronary artery disease in men and women. *JAMA.* 1996 Sep 18;276(11):875-81.
21. Watanabe H et al. Decreased high-density lipoprotein (HDL) particle size, prebeta-, and large HDL subspecies concentration in Finnish low-HDL families: relationship with intima-media thickness. *Arterioscler Thromb Vasc Biol.* 2006 Apr;26(4):897-902.
22. Thacker EL et al. Fasting and post-glucose load measures of insulin resistance and risk of ischemic stroke in older adults. *Stroke.* 2011 Dec;42(12):3347-51.
23. Johnston BC et al. Probiotics for the prevention of pediatric antibiotic-associated diarrhea (Review). *Cochrane Database Syst Rev.* 2011 Nov 9;(11):CD004827.
24. Mendall MA et al. Relation of serum cytokine concentrations to cardiovascular risk factors and coronary heart disease. *Heart.* 1997 Sep;78(3):273-7.
25. Packard RR & P Libby. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clin chem.* 2008. Jan;54(1): 24-38.
26. Fischbach FT & MB Dunning. *A Manual of Laboratory and Diagnostic Tests*, 8th Ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins, 2009.
27. Hennequin C et al. Possible role of catheters in *Saccharomyces boulardii* fungemia. *Eur J Clin Microbiol Infect Dis.* 2000;19:16-20.