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Bioavailability and pharmacodynamics of eicosapentaenoic acid and docosahexaenoic acid in soymilk and commercial supplements

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I. Objectives

The goal of this study is to assess if docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) accumulate in blood lipids to the same extent when consumed as a fortified beverage as opposed to a traditional capsule as well as assess compliance to daily consumption, tolerance and acceptability of a fortified soymilk beverage.

It is hypothesized that DHA and EPA levels will be equivalent in erythrocytes after 4 weeks of fortified soymilk consumption as compared to commercial capsules.

We propose the following objectives:

A. Evaluate the effect of EPA and DHA matrix (beverage or capsule) on accumulation of EPA and DHA in lipid pools over 4 weeks of daily consumption.
    1. EPA and DHA levels will be measured in erythrocytes, peripheral blood mononuclear cells (PBMC), platelets, and plasma over time.
B. Assess suitability of soymilk as a delivery vehicle for EPA and DHA.
    1. Compliance will be measured using daily intake logs and the return of empty and full bottles of soymilk or remaining capsules
    2. Tolerability will be monitored using a daily symptom journal.
    3. Sensory acceptability will be monitored initially and after 4 weeks of consumption to validate long term use of the fortified soymilk.

II. Background and Rationale

Research shows that fish oil and its component fatty acids EPA and DHA provide health benefits such as reducing the risk of cardiac death and lowering inflammation[1]. Yet a large portion of the population is not consuming the recommended amount of fish due to high cost, dietary restrictions such as vegetarianism/veganism, concerns about high levels of mercury, general dislike, and other factors[2]. Additionally, to meet the recommendations fish and fish oil production present sustainability challenges. A potential alternative is to utilize EPA and DHA from algae. Algae oil has performed equally to fish oil in several clinical studies and has been demonstrated as safe for consumption[3]. These sustainable oils can be added to foods increasing the potential for fatty acids to counteract chronic disease and increasing access to general consumers.

Emulsification of omega-3 fatty acids and incorporation into food products have been shown in multiple studies to enhance short-term bioavailability of the oil[4]. This pre-emulsification before digestion improves the rate and extent of incorporation into lipid pools[4]. Some longer term studies of 4 weeks showed no difference or bioequivalence between algae or fish oil capsules and various fortified foods or microencapsulated oils[5–7]. However, soymilk has not been assessed as a vehicle for EPA and DHA. Therefore, the bioavailability or bioequivalence to capsules needs to be measured. This study will offer valuable data showing that algae oil consumed in a soymilk matrix has the ability to alter lipid pools in the long term. This study will also provide preliminary data including compliance, sensory acceptability, and tolerability, validating the use of an algae oil fortified soymilk in future clinical studies.

Previously, our lab has developed, and optimized EPA and DHA fortified non-dairy plant milk beverages utilizing algae oil emulsions (food grade). Soymilk was chosen as the optimal vehicle due to its ability to mask some of the algae oil flavor, while maintaining stability of the fatty

acids. Up to 0.4% algae oil can be added to soymilk with limited changes to overall liking. Consumers did not prefer the control samples over 0.4% algae oil samples at statistically significant levels, meaning that they did not reject those samples. These samples were unflavored and addition of flavoring such as chocolate or vanilla is expected to increase sensory acceptability even more and mask algae oil flavor.

0.4% algae oil in 12 oz. of soymilk equates to approximately 700 mg of combined EPA and DHA (453 mg DHA and 249 mg EPA). In the 2020-2025 Dietary Guidelines for Americans, it was recommended to consume 8-12 oz of seafood per week, providing an average of 250-375 mg of the polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)[8]. The dietary guidelines cite the reduction in risk of cardiac deaths and beneficial effects for women who are pregnant or breastfeeding as the primary reasons for the seafood recommendation. Based on these recommendations, 700 mg of EPA and DHA should be more than enough per serving. However, many studies looking at the health benefits of EPA and DHA from fish or algae use servings of around 1 gram or higher [2,9]. Therefore, multiple servings could be given depending on the goals of the clinical trial.

This study will compare the algae oil fortified soymilk to a commercial algae oil capsule containing the same base oil with the goal of demonstrating equivalent or greater accumulation of EPA and DHA in blood lipid pools.

III. Procedures

A. Research Design

This study will compare blood EPA and DHA concentrations over time after daily consumption of algae oil capsules or algae oil fortified soymilk. The proposed study is a randomized parallel arm trial involving 24 healthy participants. The study will be open label, since participants will know if they are consuming the study agent or pill. The intervention will include a 2-week placebo run-in phase and a 4-week intervention (Figure 1) with 4 total clinic visits to the OSU Clinical Research Center (CRC).

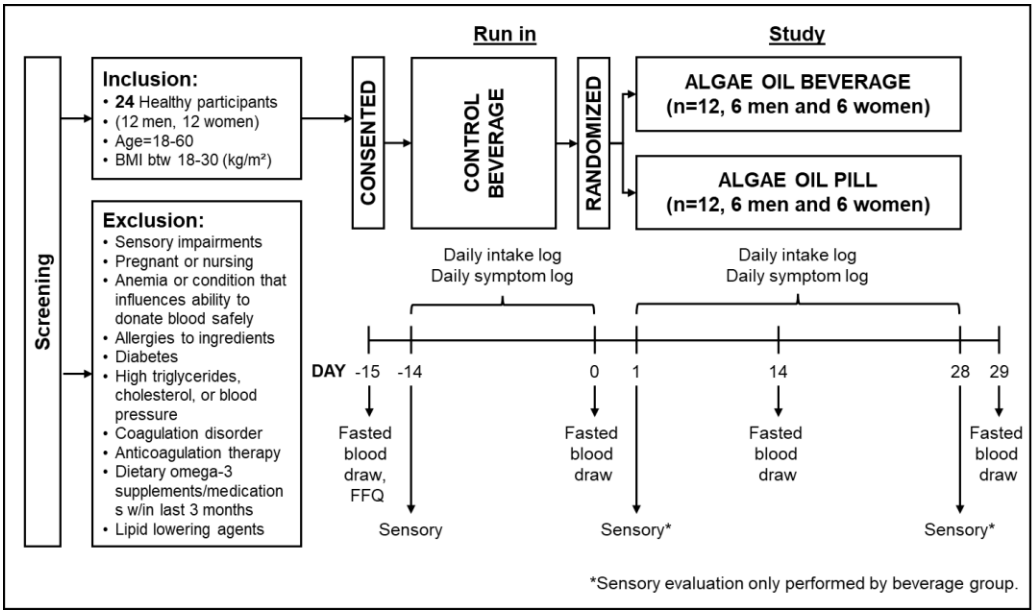

Figure 1: Study Design

B. Sample

This study will include healthy participants aged 18-60 years of age with a body mass index (BMI) between 18 and 30 kg/m^2.

Exclusion criteria will include:

- Being pregnant or nursing
- Having anemia or a condition that influences the ability to donate blood safely
- Allergies to the beverage or pill ingredients
- Diabetes
- High triglycerides or cholesterol
- Coagulation disorder
- Anticoagulation therapy or any drug that affects blood clotting.
    - The NIH states that omega-3 supplements may interact with drugs that affect blood clotting (https://www.nccih.nih.gov/health/omega3-supplements-in-depth).
- Taking prescribed dietary omega-3 fatty acid medications including fish oil or algae oil within the last 3 months
- Taking non-prescribed dietary omega-3 fatty acid supplements within the last 3 months and not willing to discontinue taking them for the study period
- Taking lipid lowering medications such as statins
- Sensory impairments which affect ability to taste, smell, or see food products

Sample size justification

- The sample size (10 per group) was calculated based on a one way ANOVA with a meaningful mean difference of 20%, power of 0.80, alpha of 0.05, and a standard deviation of 15% based on literature values of erythrocyte DHA levels [6]. The expected dropout rate is 20%. Therefore, 24 participants will be recruited.

Recruitment strategy

Informational flyers will be posted in various places across OSU's campus including the Food Science and Technology building, Howlett Hall, and Campbell Hall. Additionally, an email will be sent to the student list services including the Food Science and Technology, OSU Nutrition, and Horticulture lists. The study may also be advertised in college and departmental newsletters if needed. Emails and flyers are attached. Interested subjects will be directed to a Qualtrics survey for pre-screening to determine eligibility. Those who meet the study criteria will be contacted by the study coordinator, Abigail Sommer, to begin the informed consent process.

It is expected that subject accrual will be achieved using these methods as they have been successful previously in multiple of our sensory evaluation and clinical studies.

Pre-Screening

Interested participants will be screened through a Qualtrics survey to assess for inclusion and exclusion criteria defined in the previous sections. The Qualtrics survey is attached this application. Abigail Sommer will review the responses to determine if inclusion and exclusion criteria are met before contacting those eligible.

Informed Consent

Persons who are interested and eligible will be scheduled for a Day -15 visit with a study coordinator at the CRC. On the first visit, participants will begin the informed consent process and all study procedures will be explained. If they agree to participate, subjects will be given a copy of the informed consent form for their records.

C. Measurement and Instrumentation

1. Biological Measures

The EPA and DHA concentration in multiple blood fractions will be measured including erythrocytes, peripheral blood mononuclear cells (PBMC), platelets, and plasma.

Erythrocyte fatty acids concentration correlates better with intake and reflect longer term intake as compared to plasma [10]. Therefore, the primary objective is to determine if erythrocyte DHA and EPA levels differ after consuming algae oil fortified soymilk versus commercial algae oil capsules daily for four weeks.

A total of 24 mL of blood will be collected at each time point into two Vacutainer EDTA tubes (6 mL each) and 2 CPT tubes (8 mL each).

Blood collected into EDTA tubes will be inverted immediately following collection and then stored on ice until centrifugation (within 1 hour of collection). EDTA tubes will be centrifuged at 1932 x g for 10 minutes at 4°C. The plasma will be extracted and aliquoted into 2 mL tubes. Tubes will be stored on ice after aliquoting. The buffy coat layer will be removed and discarded, and the red blood cells will be extracted. The plasma and RBC aliquots will be stored at -80°C until analysis.

Blood collected in the CPT tubes will be prepared according to the manufacturer's instructions. The tubes will be stored upright and centrifuged within 2 hours of collection. The tubes will be inverted before centrifuging at 1800 x g for 30 minutes at room temperature. A portion of the plasma will be removed from the top of the tube and the cells will be resuspended in PBS buffer to a volume of 15 mL. The tube will be inverted to mix and centrifuged again at 300 x g for 15 minutes. The cells will be washed again with PBS. The remaining pellet will be mixed with 1 mL of PBS and then transferred to 2 mL tubes. After centrifuging again at 300 x g for 5 minutes the supernatant will be aspirated off and the cell pellet will be stored at -80°C until analysis.

Fasting blood samples at each time point will be collected into two Vacutainer tube containing EDTA and immediately stored on ice until centrifugation at 1932 x g for 10 min at 4C. Blood will also be collected in one CPT tube to be used for PBMC samples.

Blood will be separated into three fractions, plasma, PBMC and erythrocytes using previously described methods[1112]. Plasma will be stored at -80°C until analysis. PBMC and erythrocytes will be washed with buffer and hemolyzed before being stored at -80°C until analysis.

Fatty acid methyl esters in each fraction will then be separated and analyzed using gas chromatography as described previously[12]. Briefly, lipids from each fraction will be extracted with chloroform and methanol and methylated with hydrochloric acid in methanol. Erythrocyte fatty acids will be extracted and methylated using boron-trifluoride in methanol. Fatty acid methyl esters will then be analyzed using gas chromatography.

Other lipid species and metabolites including cholesterol, eicosanoids, and hormones (leptin, adiponectin, and insulin) may be measured as exploratory endpoints.

2. Clinical and Anthropometric Measures

Vital Signs

Pulse, blood pressure, and temperature will be obtained by trained, registered nurses in the CRC using standard clinical methods.

Body weight

Participants will be weighted, wearing light clothing, on a standardized scale.

Height

Height will be measured using a standardized wall-mounted scale.

Body mass index

Body mass index will be calculated using the weight in kg divided by the square of height in meters.

3. DHA and EPA Concentration in Ingredients, Beverages, and Capsules

Although the certificates of analysis are provided for the Life's Omega 60 algae oil and the algae oil capsules, we will verify the lipid profile of each to ensure standardization of the two agents.

Fatty acid profiles of algae oil capsule, Life's Omega 60 bulk algae oil (used in beverages), and fortified soymilk will be analyzed. A sterile needle will be used to extract oil from algae oil capsules. Oil from beverages will be extracted using chloroform and methanol. Fatty acids from each oil will be methylated and analyzed using gas chromatography as described previously[12].

4. Food Frequency Questionnaire

To assess diet history, especially habitual consumption of fish and shellfish or fish oil supplements, as well as plant-based milk beverages, a validated food frequency questionnaire will be used.

The Diet History Questionnaire III (DHQIII) from the NIH National Cancer Institute will be used (https://epi.grants.cancer.gov/dhq3/index.html). The study coordinators will create a study through the institute site to record and obtain data. The DHQIII includes information on 135 food and beverage items and 26 dietary supplements. The data will be collected for the past month with portion sizes.

Responses will be collected from the participants online when convenient for them during the first week of the study. Login information will be provided at the first clinic visit.

Data from the DHQIII may also be used to assess the Healthy Eating Index of each participant.

5. Sensory Evaluation

Sensory evaluation will be recorded during the first day of control beverage consumption for all panelists and during the first and last day of fortified beverage consumption for the algae oil beverage group only. Sensory evaluation will not be performed on the algae oil pills.

The sensory evaluation will include acceptability, just about right, and descriptive attribute tests as well as general impressions. The acceptability tests will include a 9-point hedonic scale from

dislike extremely to like extremely. The categories measured will be overall liking, aroma (smell), flavor (taste), texture (mouthfeel), and appearance.

The JAR tests will measure the level of serving size, chocolate aroma, chocolate flavor, sweetness, and thickness using 5-point scales from “much too little” to “much too much” of an attribute, with the middle being just about right.

The descriptive attribute test will measure their opinion on the intensity of a specific attribute including fishy aroma/flavor, beany aroma/flavor, sweetness, chalkiness, viscosity, and astringency. The scale used will measure intensity from barely detectable to strongest imaginable using a validated labeled magnitude scale.

Finally, there will be questions on general impressions including the sample size of beverages they would consume in a single day, the bottle size, and the flavor.

6. Daily Intake and Symptom Journal

A daily intake journal will be used to assess compliance. Subjects will record if and when they consumed the beverage or pill each day. Additionally, they will record If and when they consumed any of the restricted foods such as fish or shellfish and how much. The final part to the daily log will include recording symptoms such as bad breath, nausea, or bloating.

D. Detailed Study Procedures

Study Agent Information

The study agent is a flavored soymilk beverage fortified with food grade algae oil (Life’s Omega 60, DSM, Heerlen, Netherlands). The beverage has been validated by our lab to be physically and oxidatively stable over time and be acceptable in sensory evaluation studies.

The control agent will be a commercial algae oil pill (Swanson Plant Based Omega-3) containing the same Life’s Omega 60 oil. These supplements are produced in an NSF International registered facility utilizing good manufacturing practices. Additionally, their purity and potency are assessed by an independent third-party laboratory.
https://info.nsf.org/Certified/455GMP/Listings.asp?Company=C0054895&Standard=455-2GMP

https://info.nsf.org/Certified/GMP/Listings.asp?Company=C0054895&Program=DIETSUPP

All plant milk samples will be prepared in the FDA inspected Food Processing Pilot Plant (Howlett Hall) or the Dairy Processing Pilot Plant (Parker Food Science and Tech Building) using Good Manufacturing Practices. All products prepared for human consumption in this project contain wholesome food ingredients and Generally Recognized as Safe food additives. All ingredients will be food grade.

Algae oil (0.4%) will be mixed with high oleic sunflower oil, liquid lecithin, and butylated hydroxy toluene (BHT) and stirred on a hot plate set to 500 rpm and 50°C until lecithin is evenly dispersed (about 10 minutes). The water phase will be prepared by mixing 0.1% gellan gum and 1% polysorbate 80 into water using a hot plate set to 1000 rpm and 85°C. To prepare the base emulsions, 21%

Concentrated soymilk base or soymilk powder will be combined with water (to manufacturer’s instructions) sugar (2%), salt (0.1%), emulsion base (10%), and cocoa powder (2-3%) before

being homogenized, and heat treated (136°C for 4 seconds). The plant milks will be cooled and bottled in aseptic hood. All samples will be refrigerated after bottling.

Fresh soymilk will be prepared less than one week before the first week of the study and will be utilized throughout the duration of the study. Prior to this study, a confirmatory shelf-life study will be done to ensure that the storage time does not influence the fortified soymilk sensory perception. If needed, fresh samples can be prepared weekly or biweekly to ensure acceptability.

First Clinic Visit

Recruited subjects will meet with the study coordinator at the CRC to begin the informed consent process. The study coordinator, Abigail Sommer, will explain all study procedures as well as explain how to follow the low marine omega-3 diet, fill out daily intake and symptom logs, sensory evaluation forms, and the food frequency questionnaire.

If the subject agrees to participate, they will be given control beverages to consume during the run-in phase and forms including sensory evaluation, food frequency questionnaire, and daily intake and symptom logs. Finally, a preliminary blood sample will be taken from the participant to assess their usual levels of EPA and DHA in blood lipid pools before the washout.

Run-in Phase

The study will include a 2-week placebo run-in phase where subjects will consume one serving of the control beverage per day with no algae oil. During the run-in the participants will complete a daily intake log, monitoring if and when they consumed the control beverage, as well as complete a log of symptoms, and sensory evaluation on the beverage. These tasks will help familiarize them to the procedures that will be performed during the rest of the study.

Additionally, the two weeks will serve as a washout where they will be instructed to avoid a list of foods and supplements containing EPA and DHA. A food frequency questionnaire will also be used to assess usual intake of fish, shellfish, and plant-based milk beverages and healthy eating index.

Participants will be asked if they would like to receive email reminders detailing daily tasks. If so, study coordinators will send emails reminding the subjects of each daily task including drinking the beverage or taking the pills, filling out the daily dairy and applicable DHQIII or sensory evaluation tasks. Additionally, each participant will be reminded of clinic visits and instructions to come having fasted for 12 hours.

Study Phase

After the run-in, the participants will then be randomized to the beverage or pill group. During the study phase, there will be three clinic visits to the Cancer Research Center. The visits will occur on day 0, 14, and 29. At each visit, blood will be drawn.

Additionally on days 1 and 28, they will perform a sensory evaluation on the beverage using provided forms. The second sensory evaluation will be used to determine if long-term consumption of the beverage impacted its perception.

Over the course of the 28 days, participants will complete daily intake and symptom logs to ensure compliance to the low marine omega-3 diet and study intervention as well as monitor adverse effects.

To ensure compliance during the study, participants will be asked to return empty beverage or pill containers and any that were not consumed. One to three extra beverages or pills will be given to estimate how many were consumed.

Participants will again be asked if they would like to receive email reminders detailing daily tasks.

Low Marine Omega-3 Diet

Subjects will be instructed to follow a diet low in marine omega-3s including EPA and DHA. This diet will restrict the following items: fish (salmon, anchovies, swordfish, halibut, tuna, trout, sardines, cod, pollock, catfish, mahi mahi, etc.), shellfish (mussels, oysters, crab, scallops, shrimp, clams, lobster, etc.) and EPA and/or DHA enriched foods, or supplements containing fish or algae oils. If the participant does accidentally consume a restricted food, they will be asked to record the date, time, and amount of food item.

Risks and Benefits

A medical doctor, Dr. Steven Clinton, will be notified immediately of any adverse events and the study coordinator will report any serious adverse events to the OSU IRB and the OSU clinical scientific review committee.

The risk of consuming omega-3 fatty acids in the form of algae oil are extremely low. Omega-3 supplements usually produce only milk side effects such as unpleasant taste, bad breath, bad-smelling sweat, headache, and gastrointestinal symptoms such as heartburn, nausea, and diarrhea[13]. Up to 5 g of EPA and DHA supplements has been concluded to be safe by the Food and Drug Administration[13]. This study utilizes a dose well under 5 g and additional sources of EPA and DHA should be minimized in the diet due to the restrictions of the study.

Omega-3 dietary supplements can interact with certain medications including blood thinners and anticoagulants such as Warfarin[13]. During the screening process, those taking these medications will be screened out and not allowed to participate in the study, minimizing this risk.

We will assess each patient for any adverse event using the Common Terminology Criteria for Adverse Events (version 4.0) from the National Cancer Institute and National Institutes of Health. Patients who develop a grade two adverse event will be asked to stop the intervention. Although unexpected with a food product, if we see 2 similar grade three adverse events, we will stop the study and ask all men and women to discontinue the intervention.

The likelihood of serious adverse events is very low. The compounds in the foods provided are commonly found in a general, varied diet. However, any life-threatening reactions, which may be due to the beverage or supplement and all fatal events while on study or within 30 days of treatment, and the first occurrence of any previously unknown toxicity of any grade will be reported as serious adverse events. The Study Coordinator or data manager will report these reactions within 24 hours to the National Cancer Institute Investigational Drug Branch and the IRB within 10 working days. The principal investigator and the attending physician in collaboration with the NCI, if necessary, will decide if a toxicity is most likely drug related.

The benefits to the participant's health over a short period of time are unknown and likely to be small. In order to compensate participants for time and effort as well as parking and travel costs, we will provide $20 in cash for the first three clinic visits and $40 in cash for the final clinic visit and ~~$16 in cash, $4 per visits, as reimbursement for one hour of parking in the Wexner Medical Center Garage or Davis Outpatient Garage.~~ ~~parking passes as needed~~.

Subject confidentiality

Strict patient confidentiality of records will be maintained. Random 2-digit numbers will be assigned to each subject and will be used to identify all of that subject's samples and files. Individuals will not be identified by name or by any other personal identifying information in laboratory records, reports, or publications resulting from this study. Thus, risk of disclosure of confidential medical information will be essentially nonexistent. The project team has procedures in place to maintain confidentiality, such as password protected computer access, locked file cabinets and rooms where records are stored, and all team members have received appropriate training, in full compliance with HIPAA. Only the Investigators and study coordinators will have access to any subject data.

Personal data will be kept at least until publication of results, but in most cases is kept longer in case potential follow up studies with the same subject will be designed. Typically, personal information will be shredded after 10 years, and all computer files carrying personal identifying data will be erased.

Limitations and Potential Pitfalls

The capsules will utilize the same oil product (Life's Omega 60 oil, DSM) but will not contain the exact same lot. To standardize the oil as much as possible, the EPA and DHA content of the pills and beverage will be measured, with the oil concentration of the beverage being adjusted to match that of the pill if possible.

Internal and External Validity

Subjects will be stratified by gender and randomized to the study arms to enhance study internal validity. Age, BMI, and initial EPA and DHA levels will be measured in all participants to ensure even distribution across study arms. If differences in these variables are found, they will be used as covariates in data modeling.

Efforts will be made to ensure a diverse sample of study participants by recruiting from various areas around campus and not just recruiting students. Women will represent 50% of the study participants. No participant will be excluded on the basis of race or ethnicity.

Data Analysis

All data analysis will be performed using SAS statistical software.

The impact of the algae oil form on the primary outcome of change in erythrocyte EPA and DHA concentration will be evaluated using analysis of variance (ANOVA). Covariates including BMI, height, weight, sex, age, ethnicity, and pre-trial and baseline levels of EPA and DHA will be evaluated using a forward selection procedure.

The influence of product type on secondary outcomes including EPA and DHA concentrations in other blood lipid pools will also be evaluated using ANOVA.

Compliance will be measured using the daily intake log and number of empty bottles, pills received at each clinic visit. The number of days that the beverage is consumed and any noncompliance with the marine omega-3 restricted diet divided by the number of total days will be used to report compliance.

Exploratory analyses including influence of algae oil vehicle on various blood lipids, hormones, and metabolites and the influence of diet on various variables may also be performed.

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