PROTOCOL
Diet and the Colonic Exfoliome: A Novel, Non-invasive Approach to Testing Interventions in Humans

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MPI: Drs. Johanna Lampe and Meredith Hullar (Fred Hutch) and Robert Chapkin (Texas A&M)

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1. PROTOCOL SYNOPSIS

Studies in animals have shown that both long-chain n-3 polyunsaturated fatty acids (PUFA) and a bacterial metabolite of dietary fiber (butyrate) may reduce colon tumor formation, and that the two in combination are even more effective than either alone. Several different mechanisms of action have been suggested. In humans, some epidemiologic studies suggest that people who consume high-fiber diets or use n-3 PUFA supplements may have a lower risk of colorectal cancer, but there are currently no controlled dietary interventions evaluating the combined effects of these two dietary constituents on colorectal cancer risk factors in humans. We will test the combination of n-3 PUFA and fiber on gene expression in colon cells that are part of the pathways important to cancer risk and will seek to understand how variation in the gut bacterial community may affect response to intervention.

Design: A randomized, placebo-controlled, double blinded, crossover dietary intervention

Sample Size: Recruitment of 40 participants with the goal of having 30 complete study measures.

Participants: Men and women

Selection: Individuals from the Greater Seattle area who are normal to overweight (BMI of 18-30 kg/m^2) and between the ages of 50-75, who typically consume diets with 20g fiber or less per day. We will recruit U.S. racial minorities at an overall level of 32%, which mirrors the minority distribution in Seattle/King County

Exclusion: The exclusion criteria will address a mix of medical and practical issues: 1) chronic medical illness, history of gastrointestinal disorders (e.g., ulcerative colitis, Crohn disease, celiac sprue, HNPCC, familial adenomatous polyposis, pancreatic disease, previous gastrointestinal resection, radiation or chemotherapy, and cancer (other than non-melanoma skin cancer); 2) weight change greater than 4.5 kg within past year; 3) oral or IV antibiotic use within the past 3 months; 4) regular use of aspirin or NSAIDs; 5) smoking; 6) known allergy to fish; 7) intention to relocate out of study area within next 4 months; 8) abnormal liver/kidney function as ascertained at a baseline blood draw.

Intervention: a) Placebo and b) Supplemental soluble fiber (35 g/d) + supplemental n-3 PUFA (6 g/d eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

2. BACKGROUND/SIGNIFICANCE

Diet is an important risk factor for colorectal cancer (CRC) and several dietary constituents implicated in CRC are modified by gut microbial metabolism. Microbial fermentation of dietary fiber produces short chain fatty acids (SCFA), e.g., acetate, propionate, and butyrate. Dietary fiber has been shown to reduce colon tumors in animal models and, *in vitro*, butyrate influences cellular pathways important to cancer risk. Further, work from our group suggests that the combined effects of butyrate and long chain omega-3 polyunsaturated fatty acids (n-3 PUFA) found in cold-water, oily fish, may enhance the chemopreventive potential of these dietary constituents. We postulate that the relatively low intakes of n-3 PUFA and fiber in Western populations and the failure to address interactions between these dietary components may explain why chemoprotective effects of n-3 PUFA and fermentable fibers have not been detected consistently in prospective cohort studies. We hypothesize that the combined effects of n-3 PUFA supplementation and supplemental highly fermentable fiber will alter critical pathways important to CRC prevention, particularly intrinsic mitochondrial-mediated programmed cell death resulting from the accumulation of lipid reactive oxygen species (ferroptosis), cell proliferation, and the eicosanoid/inflammation pathways.

Gut bacteria ferment dietary fiber to butyrate and other SCFA.[1-14] Butyrate is a potent histone deacetylase inhibitor[15, 16] associated with reduced CRC risk.[17-19] Work from our group using preclinical models suggests that *combined effects of fiber-derived butyrate and n-3 PUFA in fish oils act synergistically* to enhance the chemopreventive potential above and beyond the contribution of butyrate alone, in part, by altering the glutathione peroxidase-4 (GPX-4)-dependent redox environment in the cell and increasing programmed cell death (ferroptosis).[20-30] These results agree with a recent prospective, nested case-control study demonstrating significant CRC risk reduction among pescovegetarians, whose diets were high in both dietary fiber and n-3 PUFA-rich fish (HR: 0.57; 95% CI: 0.40, 0.82).[31] We hypothesize that the combined effects of 35 g supplemental fiber, high in soluble fibers, and 6 g supplemental long chain n-3 PUFA (DHA + EPA) will alter pathways important to CRC prevention.

This is a randomized, controlled crossover pilot study in 40 healthy men and women (50-75 y) to compare supplemental soluble fiber (35 g/d) + supplemental n-3 PUFA (6 g/d EPA+DHA), in quantities mirroring mean daily intakes associated with lower CRC risk, with a maltodextrin and corn oil control (placebo). Stool samples will be collected at the beginning, middle (day 15), and end of each of the two 30-day intervention periods. Using a novel, cost-effective, non-invasive approach, we will evaluate differences in global gene expression signatures in the stool exfoliome (i.e., sloughed colonic epithelial cells in stool) using RNA-Seq.[32] We will focus on pathways related to colonic cell proliferation and apoptosis/ferroptosis, cell phenotype, and inflammatory response. Further, we will evaluate changes in gut microbial functional genes involved in butyrate production using droplet digital PCR (ddPCR). The collection of multiple samples over the intervention periods will provide a critical measure of longitudinal changes in response to the supplemental n-3 PUFA and fermentable fiber.

This pilot will be the first in humans to integrate and characterize the relationships between n-3 PUFA and fermentable fiber exposure that modulate programmed cell death and other CRC-related cell-signaling pathways through metabolic activities of the gut microbiome. Results of this controlled intervention will help to translate the current mechanistic knowledge from preclinical animal models to humans and to inform approaches for CRC prevention. Interrogating the stool exfoliome is a novel, cost-effective, non-invasive approach to studying effects of interventions on the human gut.

3. SPECIFIC AIMS, OBJECTIVES, ENDPOINTS:

Aim 1: To test in a pilot feasibility study, in humans, effects of supplemental dietary fiber (35 g) and EPA+DHA (6 g) vs placebo on mRNA expression in exfoliated colonic cells. We hypothesize that fiber and n-3 PUFA supplementation will uniquely influence colonocyte proliferation and programmed cell death-related gene expression and signaling pathways.

Aim 2: To test whether response to the intervention differs by gut microbial functional capacity related to fiber fermentation. We hypothesize that changes in colonocyte gene expression will be modified by the

gut microbial capacity to ferment dietary fiber (i.e., SCFA-producing pathways), measured as butyrogenic potential. ∑(butyryl-CoA:acetate CoA-transferase (*but*) + butyrate kinase (*buk*) genes)/(total bacterial load 16S rRNA gene) assayed using digital drop PCR.

Objectives and Endpoints:

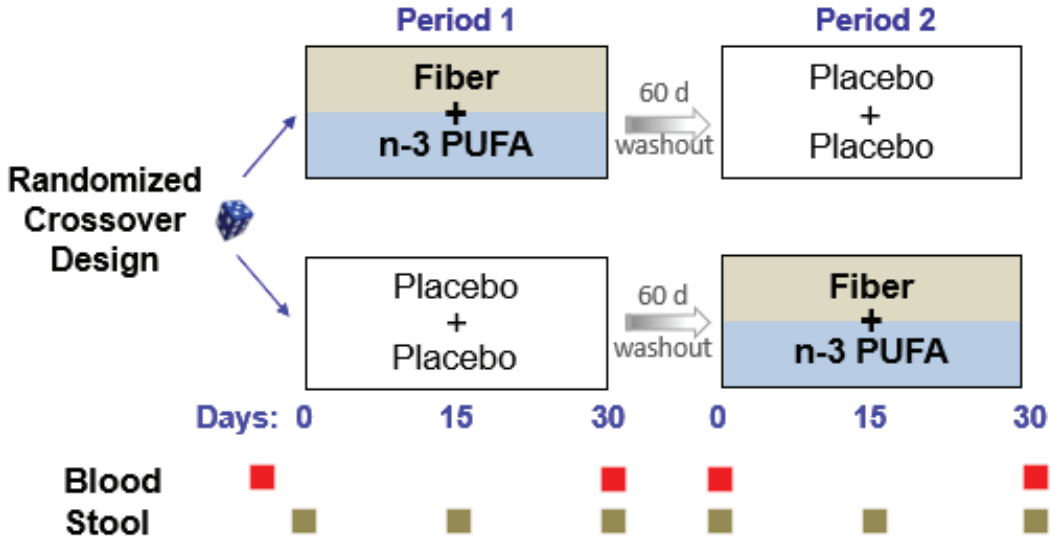
1. To determine whether the combination of supplemental dietary fiber and EPA+DHA vs placebo affects mRNA expression in exfoliated colonic cells. The endpoint is mRNA expression profiles in the exfoliome.

2. To determine whether gut microbial functional capacity related to fiber fermentation, as measured by presence of fiber-fermenting genes in stool bacteria, alters exfoliome mRNA expression response to the intervention on. The endpoint is mRNA expression profiles in the exfoliome.

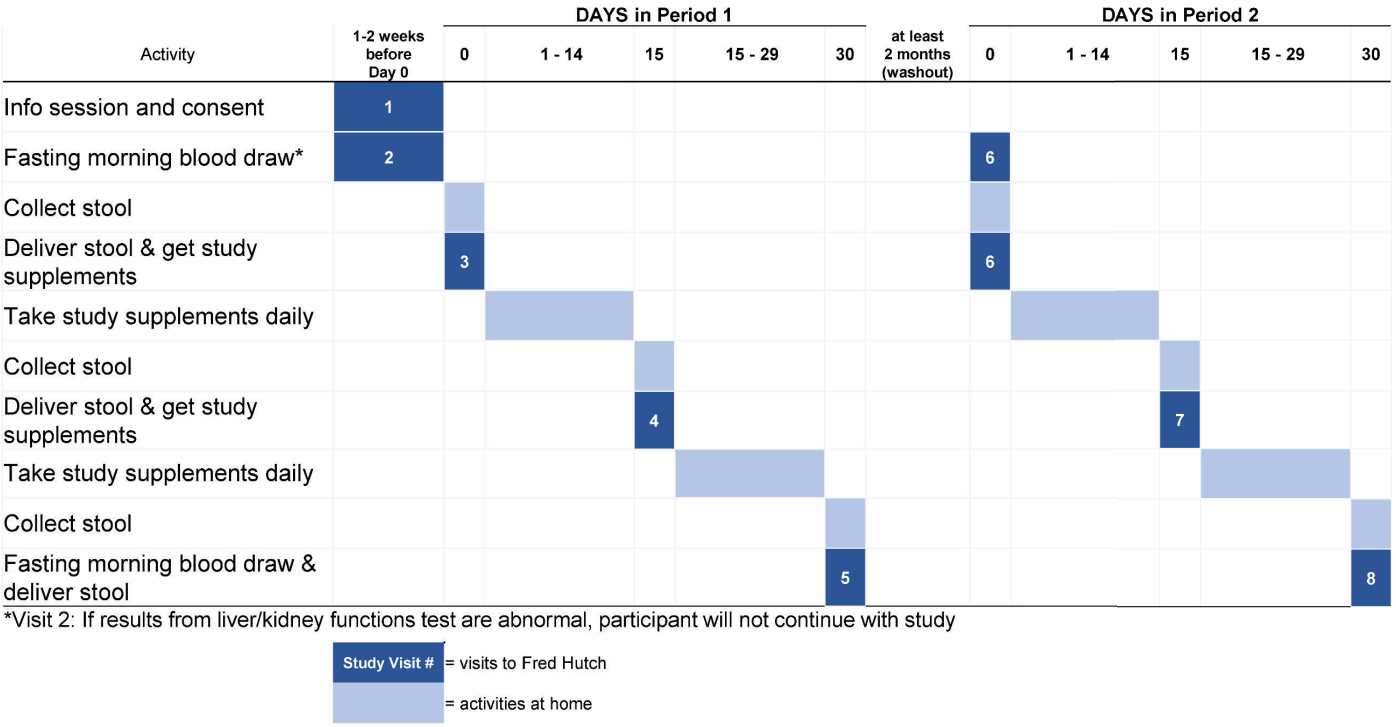
Implications: This proposed pilot feasibility study will be the first controlled study in humans to integrate and characterize the relationships between n-3 PUFA and prebiotic fiber exposure that modulate programmed cell death/CRC-related cell-signaling pathways through changes in the gut microbiome. Results of this human mechanistic controlled intervention will help to translate the current mechanistic knowledge from preclinical animal models to humans and to inform approaches for CRC prevention. Also, importantly, we will further evaluate the utility of interrogating the exfoliome as a novel, non-invasive approach to studying effects of interventions on the human gut.

4. STUDY DESIGN

The study is designed as a randomized, cross-over, placebo-controlled dietary intervention of supplemental soluble fiber and supplemental n-3 PUFA compared to corn oil and maltodextrin control. We will randomize participants to one of the two treatment orders, either supplemental fiber and fat first followed by the placebo in the second period or the placebo first followed by supplemental fiber and fat. Each treatment will be 30 days in duration with at least a 60-day washout period between treatments. Stool samples will be collected at the beginning, middle (day 15) and end of each of the two 30-day intervention periods.



It will take participants at least 4 months to complete the trial. Details of the activities and timeline:



*Visit 2: If results from liver/kidney functions test are abnormal, participant will not continue with study

4.a. Fiber and Fat Supplements The active treatment period will include supplemental soluble fiber (35 g/d) + supplemental n-3 PUFA (6 g/d EPA+DHA).

Supplemental Fiber: The soluble fiber with known fermentation profiles will be provided by Tate & Lyle, a well-established food and beverage ingredient company. For placebo, we will use maltodextrin provided in the same packaging and by the same company.

Supplemental n-3 PUFA: We will use a commercial fish oil supplement (Omega Cure, Omega3 Innovations), consumed as 2 vials daily. Each vial (11.75 g oil) delivers 3 g EPA +DHA in a 1:1 ratio. For placebo, we will use equivalent amounts of corn oil in visually identical packaging provided and by the same company.

All study supplements will be in packaged as daily doses. At the study visits at the beginning and mid-point of each study period, participants will receive a box with all the supplements needed to cover the time in between visits along with instructions regarding proper storage conditions (ie. the fish oil is to be refrigerated for better preservation.)

4.b. Safety and Adverse Effects Supplemental fiber (30-45 g/d) has been well-tolerated in short and long-term interventions (4 wks to 1-10 y).[33-36] In a study specifically designed to evaluate tolerance, individuals were given 10, 30 and 60 g/d of supplemental fiber for 7 d in addition to their regular diet. All doses were well-tolerated.[37] Further, the specific product we propose to use, PROMITOR® Soluble Corn Fibre (SCF), has been well tolerated by healthy volunteers at 65 g/d.[38, 39] SCF, a glucose oligosaccharide, containing a mixture of α 1-6, 1-4, 1-3, and 1-2 glucosidic linkages, has demonstrated prebiotic effects.[40, 41] Testing of SCF in an *in vitro* SHIME method that mimics human digestion showed that SCF is well fermented in the distal colon leading to positive effects on the GMC, and that these effects are translated to immunomodulatory properties and improved gut barrier.[42] Long-term trials (6-27 months) designed to evaluate safety of supplemental n-3 PUFA up to 7 g/d have reported no adverse in general, and in bleeding complications specifically, even among patients at higher risk,[43, 44] There is insufficient data to establish a tolerable upper intake for n-3 PUFA for any population group.[45]

4.c. Length of intervention periods and washout: Description and rationale. Intervention Periods: Crypt turnover is 3.5 d and 4.2 d for the colon and rectum, respectively[46] and dietary n-3 PUFA are

incorporated into colonic cell membranes within 7 d.[47] 1-mo interventions with diet or aspirin result in measurable changes in colonocyte gene expression[48] and down-stream colon biomarkers.[49] Gut microbiome changes occur in 2-3 wks.[3, 50, 51] Changes in gut mucosal cancer-risk biomarkers, the microbiome, and microbial metabolites were also seen in a short-term 2-wk intervention of fat and fiber.[52] We hypothesize that changes in the gut microbiome influence colonic gene expression, thus conservatively we are using a 30-d period to allow sufficient time for both outcomes to change, while considering participant burden. Washout: The 60-d washout will ensure that residual intervention effects from the previous period are eliminated. As mentioned, the gut epithelium and microbiome change rapidly, but blood DHA+EPA clearance requires 45 d.[53]

4.d. Monitoring and assessing adherence. Participants will meet with study staff routinely to assess any difficulties with study adherence. n-3 PUFA are rapidly incorporated into colonic tissue.[54, 55] and there is high correlation between membrane PUFA and plasma phospholipid (PLFA) PUFA.[56, 57] We will assess adherence to n-3 PUFA supplementation using plasma PLFA profiles (**Section C.4.a**) and by monitoring vial counts, calculating adherence % (vials supplied - vials returned/days elapsed).[58] Adherence to supplemental fiber will be monitored by study staff. Participants will be instructed to return unconsumed fiber packets for weighing and consideration in intake determination. During the intervention, participants will complete daily forms to monitor adherence to the supplements.[32]

4.e. Summary of study activities and measures

Table 1. Study Activities and Measures	Reason for Measures
Screening	
Screening questionnaire	Exclusion criteria
Information session	Informed consent
Baseline blood sample to Quest Diagnostics	Exclusion criteria (liver and kidney functions)
Health and Demographics questionnaire	Exclusion criteria
Anthropometrics	Exclusion criteria
Two Supplementation Periods **Each 30 days each** **Cross-over (order randomly assigned) ***	
Stool samples (6 total) Beginning, middle and end of each period	
For colonocytes	Global gene expression signatures in the exfoliome (using RNA-Seq)
For Microbial	Microbial DNA extraction, ddPCR and SCFA.
Bowel Habits & General Symptoms questionnaire	Intervention effects
Blood samples (4 total) Baseline & end of each period	Plasma phospholipid PUFA
Weight, Baseline & end of each period	Monitor biological changes
Daily calendar	Compliance

* In between feeding periods there will be at least 60 days washout period.

5. STUDY POPULATION/RECRUITMENT

Participants will be healthy, non-smoking men and women, ages 50-75 y, who are normal to overweight [body mass index (BMI) ≥18 and ≤30 kg/m^2]. Women will be postmenopausal, with no menstrual period in 12 months. We will place ads in newspapers, distribute flyers, and use social media. A screening survey will determine eligibility (age, health, medications, habitual fiber intake); individuals will meet with the study coordinator for an info session and to obtain consent. We will measure height and weight (for BMI) and draw blood (Complete Blood Count (CBC) and Comprehensive Metabolic Panel) analyzed by Quest Diagnostics, a CLIA certified lab) will further determine eligibility.

Study information will be available online. The screening questionnaire will be downloadable. Interested persons will fill it out and send it via mail. A contact phone number and email address will also be provided for individuals who want to be mailed an information packet. Interested persons will return the completed questionnaire to the study staff, who will determine eligibility and will contact eligible persons

by phone or email to schedule a meeting. A packet containing a confirmation letter and the screening study consent form will be sent to the person prior to attending the meeting. Participants eligible to enter the intervention will meet with our staff to discuss all the details and expectations of the study, review informed consent and answer any questions they might have, and if still interested sign the consent form.

6. ELIGIBILITY CRITERIA

Inclusion: Healthy men and women aged 50-75 years who are normal-overweight (BMI of 18-30 kg/m^2), non-smoking and consume fiber intakes of less than <20 g/d.

Exclusion:

1. medical conditions: hypertension requiring medication; chronic liver disease (cirrhosis, hepatitis); kidney dysfunction or disease; previous resection of stomach or intestines; history of gastrointestinal disorders: colorectal cancer or other gastrointestinalcancer, polyposis syndromes, inflammatory bowel disease (ulcerative colitis, Crohn disease), intestinal blockage requiring surgery, pancreatic disease, radiation treatment to the abdomen within past 5 years; cancer (other than non-melanoma skin cancer);
2. regular use (more than once per week) of: NSAIDs (aspirin, ibuprofen, naproxen), Coumadin (warfarin), Eliquis (apixaban), Plaviz (clopidogrel), Xarelto (rivaroxaban), Lovaza™ (prescription fish oil), blood clotting medications;
3. weight change greater than 4.5 kg within past year;
4. oral or IV antibiotic use within the past 3 months;
5. consumption of three or more alcoholic beverages a day;
6. smoking or use of cannabis products within the past year;
7. known allergy to fish or fish-containing products; allergy to Vitamin E analogues
8. intention to relocate out of study area within next 4 months;
9. abnormal clinical lab tests as ascertained at a baseline blood draw:

Ineligible if results fall outside of the following parameters based on Complete Blood Count (CBC) and Comprehensive Metabolic Panel performed by Quest Diagnostics (a CLIA certified lab).* Blood from the baseline fasting blood draw will be sent to Quest with Study IDs not names. Parameters:

White blood cell count 3,000-11,000/mm^3
Platelet count 150,000-400,000 mm^3
Bilirubin 0.2-1.3 mg/dL
AST 0-35 U/L
ALT 0-40 U/L
Alkaline phosphatase 20-125 U/L
Creatinine ≤ 1.2 mg/dL
Potassium 3.5-5.0 mmol/L

*We will provide participants with a copy of their lab results if these are out of the normal range – which will make them ineligible for the study. We call them with this information prior to sending them the results via mail. We will encourage them to contact their physician.

7. SPECIMEN AND DATA COLLECTION

7.a. Blood We will obtain 10-hour fasting blood samples at the beginning and at the end of each intervention period. The first sample will be sent to QUEST Diagnostics (a CLIA certified lab) for clinical chemistries (to confirm eligibility; see Section 6 for specifics). Participants will come to the Prevention Center at the Fred Hutch where certified personnel will collect four 10-ml vacutainers of blood; for plasma and serum. Specimens will be stored at -80ºC.

7.b. Stool samples at three timepoints in each intervention arm, beginning, middle and end; for : a) exfoliated colonocyte transcriptome (mRNA) analysis, and b) fecal microbiome analyses. Participants

will collect small samples of stool into vials we provide that are prefilled with RNAlater for microbial DNA,[59] RNAshield for exfoliated colonocyte transcriptomics,[60] and in 95% ethanol for SCFA and future metabolomics measures.[61] Human transcriptome profiles are stable for at least 60 h at 4°C prior to addition of RNAshield solution and once RNAshield solution is added, at -80°C for at least 2 yrs.[48, 60]

7.c. Questionnaires: Dietary Assessment We will use an established, validated food frequency questionnaire (FFQ) to characterize food and nutrient intakes of the study participants over the 3 months prior to baseline to characterize habitual diets (typically low in n-3 PUFA and fiber and high in n-6 PUFA). **Health and Demographics** will also be collected. A **General Symptoms and Bowel Habits Questionnaire** will also be provided every time participants collect stool- this is a useful tool to determine intervention effects.

8. LABORATORY PROCEDURES

8.a. Plasma n-3 PUFA Assays To confirm adherence to n-3 PUFA exposure, we will isolate total phospholipids from plasma collected on days 0 and 30, trans-esterify, and measure EPA and DHA by GC/MS.[62] This work will be performed by our Co-Investigator Dr. Robert Chapkin at Texas A&M.

8.b. Host Gene Expression Using RNA-Seq Poly A^+ RNA will be isolated for exfoliated cells in stool as we describe.[48, 60, 63] An Agilent 2100 Bioanalyzer will be used to assess integrity of fecal poly A^+ RNA as we described.[64, 65] This work will be performed by MPI, Dr. Robert Chapkin at Texas A&M.

8.c. Fecal Microbiome Analysis Using ddPCR We will extract genomic DNA from stool samples and use the QX200 ddPCR system (Bio-Rad) to quantify bacterial genes from the two major butyrate synthetic pathways, butyryl-CoA:acetate CoA-transferase (*but*) and butyrate kinase (*buk*) and total bacterial load (16S rRNA genes) according to the manufacturer's instructions, and primer and probe set optimized for the selected genes.[66-70] We have chosen this assay because it is more reliable, accurate, precise, and less prone to inhibition than qPCR. Butyrogenic potential of the microbiome will be defined as ∑(butyryl-CoA:acetate CoA-transferase (*but*) + butyrate kinase (*buk*) genes)/(total bacterial load 16S rRNA gene). [66-69] This work will be performed in the JLampe lab at Fred Hutch.

9. DATA ANALYSIS

9.a. Data entry and quality assurance procedures. We will code and enter data from questionnaires, tracking forms, etc., and integrate with laboratory data, and monitor adherence to protocols for data entry, sample processing, storage, and analysis.

9.b. Aims testing, Overview: In **Aims 1 and 2** our analyses will investigate the response to the fiber and n-3 PUFA intervention at levels of **a) individual genes or variables** and **b) pathways and networks.** At the individual marker level (a) we will test genes and the butyrogenic potential (Aim 1 and 2) for differences due to intervention using univariate models, adjusting for appropriate covariates. At the pathway/network level (b), analysis will focus on hypothesized CRC pathways and employ gene-set ("global") enrichment analysis tests to evaluate changes in host gene pathways (Aim 1). When prior genetic/metabolic network structure is known within a pathway, the analysis will account for this.

Models: a) For single-gene/variable analyses (Aims 1 and 2), we use linear mixed models with a random effect for participant to account for the repeated measures of marker y during and at the end of each intervention. Fixed effects will include an indicator, *I*, for intervention as well as demographic covariates (e.g., age, sex, BMI) which will be tested by randomization and included where appropriate. Although randomization to intervention sequence should alleviate the potential for *differential* carryover effects between periods, models will include an indicator *S* of randomization sequence. Error terms ϵ_{ik} are assumed to be correlated within, and independent between, participants. In summary, models take the form $y_{ik} = \alpha_i + I_{ik} + S_i + \varepsilon_{ik}$, where α_i is a random effect for subject. Robust standard errors will be used to test for differential effect of intervention, and all p values will be corrected for multiple testing by controlling the FDR using Benjamini-Hochberg[71]. **b)** For pathways, we will apply kernel association testing (KAT) procedures.[72, 73] These multivariate methods allow for pathway-wide testing and modeling in a manner which extends gene-set style tests. They incorporate adjustment for covariates and generalize tools such as the globalTest,[74] used in genomics studies, and perMANOVA,[75-77] common to microbiome analyses. Network or structural relationships (e.g., metabolic) between genes in a pathway

are easily incorporated into construction of a similarity/kernel matrix on which KATs are based[72, 78]. These known (or estimated) interactions can enhance the KAT testing procedure to increase power.[79, 80] Similar methods analyzing gene-gene networks have been successful in crossover dietary studies.[81] We will account for the repeated-measures aspect of the crossover design in these models by applying a variant of family-based KAT.[82, 83] Finally, longitudinal data collected on days 0, 15, and 30 of each period will be analyzed using a linear mixed model for each measure, as above. Terms for time and effect modification by intervention (time*intervention) will be added to this model if the data support autocorrelation within a measure across time. For longitudinal analyses of multivariate data (i.e., pathways/ gene-sets), we will test for longitudinal differences due to intervention by way of a repeated-measures KAT[82, 83] and a variant of GSA.[84] We will consider effects of butyrogenic potential (defined as above and below the median; **Aim 2**) on host gene expression responses (**Aim 1**).

9.c. Power and sample size. This is a randomized, controlled crossover pilot feasibility study in 30 healthy men and women, designed to generate preliminary data to inform a larger human mechanistic study. Based on our previous experience, we are confident we will detect gene expression changes with the fiber + n-3 PUFA intervention. **Aim 1:** In our FlaxFx study[32] with a similar crossover design, we detected differential expression of 32 genes between placebo and lignan extract (FDR<0.05) in exfoliated cells (n=42 participants). Given the impact of dietary fiber on multiple pathways in animal models,[85] we anticipate similar or better power in the current study. In addition, we also considered power for single-gene analysis in this crossover study (n=30). Using a 2-sided paired t-test at significance level alpha=0.05 and a range of effect sizes, conservative estimates are: power of at least 80% for effect sizes larger than 0.5. **Aim 2:** a power estimate for ddPCR data, based on differences in *but* gene abundance with low- ($4.96*10^{11}$ ± $3.22*10^{10}$ copies/g feces) and high-fiber diets ($1.37*10^{12}$ ±$1.47*10^{11}$) in healthy individuals (n=30),[86] at significance level alpha=0.05 and effect sizes larger than 0.8 , we estimate power at least 95 % to detect diet-related differences in this gene. We anticipate a similar distribution for *buk*.

10. PROJECT TIMELINE

	Year 1				Year 2			
Quarter	1	2	3	4	1	2	3	4
Start up, Forms, IRB	■							
Recruitment & Intervention		■	■	■	■			
Bacterial DNA extractions and ddPCR				■	■	■		
Exfoliated Cell Assays, Global gene expression signatures in the exfoliome (using RNA-Seq)					■	■	■	
Plasma phospholipid PUFA						■		
Data Analysis and Manuscript Preparation						■	■	■

11. STRENGTHS AND LIMITATIONS OF PROJECT

The strengths are: (i) translation and testing of a hypothesis in humans that has been well characterized in animal models and is supported by cogent mechanistic evidence; (ii) a robust, randomized crossover study design with within-person pairwise comparison; (iii) feeding of n-3 PUFA and prebiotic dietary fiber[40, 41] at levels relevant to CRC prevention; (iv) pathway-based evaluation; (v) novel, non-invasive approach to measure gene expression using the stool exfoliome; (vi) targeted measures of butyrogenic potential; and (vii) extensive experience and expertise of our research team in all aspects of the proposed work. Limitations relate to the inherent challenges of short-term human dietary interventions without disease as an outcome and the R21 budget constraints. The latter limits the sample size, number of treatments (e.g., no test of n-3 PUFA and fiber individually), and comparison of exfoliome and biopsy gene expression. However, our cost-effective, robust study design, relevant dosing, and pathway-related outcomes will inform the utility of further work in this area. Further, we focus on

pathways that show, in animal models, that fish oil/fermentable fiber-containing diets protect against colon cancer by up-regulating ferroptosis/apoptosis and suppressing proliferation and inflammatory mediators.[21, 23, 24, 87-89]

12. ANTICIPATED RESULTS, ALTERNATIVE APPROACHES AND FUTURE RESEARCH

We anticipate generating strong pilot data on the utility of n-3 PUFA + fermentable fiber to affect colonic pathways relevant to CRC prevention. With our experience in conducting human intervention studies and analyzing the proposed specimens, we do not anticipate any difficulties completing this small, pilot intervention. Furthermore, an understanding of the inter-individual differences in butyrate and n-3 PUFA disposition may inform development of tailored diet strategies for CRC-risk reduction. Specimens collected as part of this study will also be stored for additional microbiome and metabolomic analysis as future funding permits.

13. DATA SAFETY AND MONITORING PLAN

Oversight for this study will be provided by the Principal Investigators, Drs. Johanna Lampe and Meredith Hullar (Fred Hutch) and Robert Chapkin (Texas A&M), with delegation of responsibilities to designated study personnel. They will ensure all entry criteria are met prior to the initiation of the protocol, and all study procedures and reporting of adverse events are performed according to the IRB-approved protocol.

Dr. Rachel Issaka, a board certified gastroenterologist at SCCA and UW Medical Center and a health services researcher with the Fred Hutchinson Institute for Cancer Outcomes Research will serve as medical advisor for the study. She will be available for consult in adverse events cases.

All adverse events related to the study procedures will be fully documented on the appropriate case report form(s) and entered in a study database. For each adverse event, the investigator will provide the onset, duration, intensity, treatment required, and outcome, including documentation of need for premature termination of any study procedures.

Anticipated adverse events related to this study are:

Blood Draws Participants may experience a little discomfort or have a temporary bruise from having blood drawn. Occasionally a participant may feel lightheaded or feel faint when having blood drawn. Blood will be taken only by trained personnel in the Fred Hutch Prevention Center Shared Resource Research Clinic using established procedures. The participants will be sitting down when the blood is drawn. If a participant feels faint, they will be instructed to lie down until the feeling goes away.

Dietary Supplements We will provide two dietary supplements during the active intervention and similar controls during the placebo periods. All participants will go through both intervention periods. Fiber: ~35 g highly-soluble, well-tolerated commercial fibers. Supplemental fiber (30-45 g/d) has been well-tolerated in short- and long-term interventions (4 wks to 1-10 y). Control: We will use maltodextrin (a food-ingredient polysaccharide made from partially hydrolysed starch) provided in the same packaging. Mild bloating and abdominal distension might occur while on the fiber treatment since the fiber content will be higher than their habitual diet. Participants will be monitored by the study staff. Most people adapt to the higher fiber intake within 3-5 days. Fish Oil: n-3 PUFA: We will provide a daily dose of 6 g EPA+DHA, consumed as 2 vials daily for 30 d. Control: We will use equivalent amounts of corn oil in visually identical packaging. Although generally safe, high amounts of EPA and DHA may thin the blood (increase clotting time) and slightly lower blood pressure. Fish oil may cause belching, bad breath, heartburn, nausea, loose stools. Participant will be instructed to report all symptoms. Study staff will contact participants after the first week on each study period to ascertain compliance and ask about any concerns they may have. Study staff will see the participants after 2 weeks provide them the needed supplements and will, again, ascertain compliance and ask about any concerns they may have. We also will maintain text and email contact with them. They fill out a daily form that includes space for commenting about symptoms or issues; thus, we are able to address the issues as they present themselves and are able to suggest ways to ways to minimize these effects. Fish Oil - Risk of allergic

reaction will be minimized through eligibility questions addressing history of medication, food allergies / intolerances. Taking fish oil supplements with meals can often decrease gastrointestinal side effects. Participants will keep a daily study log which will include questions on health status and possible adverse experiences. A general symptom questionnaire will be administered at baseline and each subsequent visit. If there are any indications that a participant has a food allergy that was not previously known, we immediately have them stop the intervention. The study coordinator has an emergency cell phone and can be reached 24/7 by study participants.

Adverse event grading scale, based on Common Terminology Criteria for Adverse Events (CTCAE) v5.0 (Publish Date: November 27, 2017):

Grades Grade refers to the severity of the AE. The CTCAE displays Grades 1 through 5 with unique clinical descriptions of severity for each AE based on this general guideline:

Grade 1 Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

Grade 2 Moderate; minimal, local or noninvasive intervention indicated; limiting age appropriate instrumental ADL*.

Grade 3 Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL**.

Grade 4 Life-threatening consequences; urgent intervention indicated.

Grade 5 Death related to AE.

Activities of Daily Living (ADL) *Instrumental ADL refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc. **Self care ADL refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

Plan for unanticipated AE reporting: All unanticipated AEs related to the study procedures that are severe or serious will be reported by Dr. Johanna Lampe the IRB within 10 days of notification of the investigator. We will use as a guide the Adverse Events Relatedness Reference Sheet (Cancer Consortium Clinical Research Support) see Appendix 2. And we will follow:

Hutch IRB Policy 2.6

Adverse Biomedical Events

When, in the opinion of the principal investigator, or PI, any adverse event meets all three of the following criteria, whether they occur on- or off-site, they must be submitted to the IRB within 10 calendar days.

- **Unexpected:** An adverse event is unexpected when its nature (specificity), severity or frequency are not consistent with (a) the known or foreseeable risk of adverse events associated with the research procedures described in the protocol-related documents, such as the IRB-approved research protocol, informed consent document and other relevant sources of information such as product labeling and package inserts; and are also not consistent with (b) the characteristics of the subject population being studied, including the expected natural progression of any underlying disease, disorder or condition, or any predisposing risk-factor profile for the adverse event.
- **Related or possibly related to the research:** An adverse event is related or possibly related to research procedures if, in the opinion of the PI, it was more likely than not caused by the research procedures. Adverse events that are solely caused by an underlying disease, disorder or condition or by other circumstances unrelated to the research or any underlying disease, disorder or condition are not "related or possibly related." If there is any question whether an adverse event is related or possibly related to the research, report the adverse event.

Serious: An adverse event is serious when it results in any of the following outcomes: death, risk of death, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity/or change in psychosocial status, a congenital anomaly or the requirement of an

intervention to prevent permanent impairment or damage. **Plan for anticipated AE reporting**: All serious anticipated AEs related to the study procedures will be reported by Dr. Johanna Lampe to the IRB within 10 days of notification of the investigator.

Plan for ongoing review of results: The PIs will be notified within 24 hours by the research manager of any early terminations due to an adverse event.

Plan for safety review: the PI will perform a cumulative review of all adverse events and premature terminations review every 6 months after study initiation or after completion of 50% of participant visits, whichever occurs first.

Plan for annual reporting: A summary of the investigation including all adverse events and how they were handled, enrollment, drop-outs and reason for discontinuation and any protocol modifications will be provided to the IRB on an annual basis.

Annual Reports will contain:

- The number of adverse events and an explanation of how each event was handled
- The number of complaints and how each complaint was handled
- The number of subject withdrawals and an explanation of why the subject withdrew or was withdrawn
- The number of protocol deviations and how each was handled

The occurrence of any serious and unexpected event may prompt changes in study protocol. Any such change will be approved by the IRB before implementation.

Monitoring for data integrity and safety will be the responsibility of all the investigators. The following will be included:

Validity and integrity of data: Data are checked for missing, unusual, or impossible records when they are entered into the study's computer database. The investigators may consider modifications to the data-collection protocol with permission from IRB if they deem the change(s) is necessary to ensure the validity and integrity of data collected.

Enrollment rate relative to expectation: Early lags in recruitment will be rectified with increased recruitment efforts so that recruitment will be completed on time. The investigators will monitor this closely to ensure full enrollment of appropriate participants.

Retention of participants and adherence to protocol: The investigators will monitor retention and adherence to study protocol. Adherence to the study supplements will be monitored via the Daily Record that includes a calendar where participants mark daily capsules consumed.

Oversight for this study will be provided by Dr. Johanna Lampe (corresponding MPI on this grant) with delegation of responsibilities to the Study Manager, Lisa Levy. They will ensure all entry criteria are met prior to the initiation of the protocol, and all study procedures and reporting of adverse events are performed according to the IRB-approved protocol. All adverse events related to the study procedures will be fully documented on the appropriate case report form(s) and entered in a study database. For each adverse event, the investigator will provide the onset, duration, intensity, treatment required, and outcome, including documentation of need for premature termination of any study procedures.

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APPENDIX 1
Study supplements – Manufacturer Product Information

OMEGA3 INNOVATIONS

Omega Cure® Environmental Analysis

Here at Omega3 Innovations, we take the quality of our oil very seriously. Every batch of Omega Cure is purified and analyzed to ensure the oil is safe and that it meets the standards for contaminants and oxidation. Below, you can see the values from a recent Omega Cure analysis, which provides an example of the typical results we achieve. In addition, you'll find the values of three international standards for omega-3 supplements for comparison purposes.

	Omega Cure Batch Sample	IFOS 5 star*	GOED Monograph*	EU Legislation Ph. Eur.*
OIL COMPOSITION				
Amount of EPA (area%)	15	-	-	-
Amount of DHA (area%)	15	-	-	-
Ratio of EPA/DHA	1	-	-	-
Total omega-3 (area%)	37	-	-	-
OXIDATION PARAMETERS				
Peroxide value (mEq/kg)	0.2	3.8	5.0	10
Anisidine value	1	15	20	30
Totox	2	19.5	26	N/A
Acid value (mg KOH/g)	0.1	2.3	3.0	-
ENVIRONMENTAL PARAMETERS				
Arsenic (mg/kg)	0.003	N/A	0.1	N/A
Cadmium (mg/kg)	<0.001	N/A	0.1	N/A
Mercury (mg/kg)	<0.001	N/A	0.1	0.5
Lead (mg/kg)	0.002	N/A	0.1	0.1
PCBs (209 Congeners) (mg/kg)	0.005	0.045	0.09	0.2
Dioxins + Furans (PCDD + PCDF) (pg/g)	0.3	1	1.75	1.75
Dioxin- like PCB's (pg/g)	0.2	1.5	3	N/A
Dioxins +Furans + Dioxin-like PCB's (pg/g)	0.5	N/A	3	6.0
Sum PAH4 (µg/kg)	ND**	N/A	N/A	10
Benzo(a)pyrene(µg/kg)	<0.5	N/A	N/A	N/A
Pesticides (mg/kg)	ND**	-	-	-

* **Maximum** recommended values. ** **ND** is an abbreviation, meaning "non-detectable."

TATE & LYLE

Specification Sheet **300000001508**

PROMITOR™ Soluble Corn Fiber 85A

1810012115021, 1810012115003, 18100121

Product Description : PROMITOR™ Soluble Corn Fiber is a versatile soluble fiber that imparts no color or off flavor in a wide variety of food and beverage applications.

Appearance: Powder
Color: Off-White
Odor: Starch

Solubility Statement: Soluble In Water

Quality Inspection Plan (Official Specification Values)

Parameter	Target	Range/ Attribute	Unit	Method
SEDIMENT		<= 16	ppm	Sediment - TN70570
COLOR		<= 2.5		Color - TN22505
MOISTURE		<= 6.00	%	Moisture - TN46015
pH		3.5 - 5.5		pH - TN60520
FIBER		>= 85.0	%ds	Repolymerized Compounds - TN65645
SCREEN ON US#20		<= 10.00	%	Screen Size - TN69700
SCREEN ON US#100		>= 60.00	%	Screen Size - TN69700
SUGARS, AOAC		<= 2.0	%	Sugars, AOAC - TN67475
DENSITY		20.00 - 24.00	lb/scf	Bulk Density - TN24551
SOLUBILITY		<= 30.0	s	Solubility - TN76000
BACTER TOTAL		<= 1000	n/g	Total Plate Count - TN10560
OSMOPHYLIC YEAST		<= 50	n/g	OSMOPHILIC MOLD - TN10525
OSMOPHYLIC MOLD		<= 50	n/g	OSMOPHILIC MOLD - TN10525
COLIFORMS		<= 3	n/g	Coliforms - TN10510
E. COLI		None Detected		Coliforms - TN10510
SALMONELLA		Negative		Salmonella - TN10547

Product Composition / Labeling

Labelling regulations may vary from one country to another, please consult with your own regulatory personnel when determining how to label your finished product.

If you require any further information on our ingredient, please contact your local Tate & Lyle representative.

Regulatory Status

Compliance Statement:
- Complies with US FDA Regulation 21CFR184.1444 - generally recognized as safe.

21 CFR References: 21CFR184.1444

Country of Origin

Country of Manufacture: United States

Kosher Status

Certified Kosher: Yes
Certified Kosher for Passover: No

The above-mentioned ingredient or its components is certified Kosher by:
- Orthodox Union (OU)

Halal Status

Certified Halal: Yes

Vegetarian Status

Vegetarian: Suitable
Vegan: Suitable
Ovo-Vegetarian: Suitable
Lacto-Vegetarian: Suitable
Lacto-Ovo-Vegetarian: Suitable

Presence of Allergens

If present in this Tate & Lyle ingredient, allergens will be denoted as “Present” in the table below.

Allergen	Status
Crustacean & Shellfish	Absent
Mollusk	Absent
Fish	Absent
Egg	Absent
Milk (including Lactose)	Absent
Peanut & Oils	Absent
Soybean & Oils	Absent
Gluten Containing: Wheat, Rye, Barley, Oats, Spelt,	Absent

Kamut, Triticale	
Seeds, Sesame & Oils	Absent
Celery/Celeriac	Absent
Mustard	Absent
Lupin	Absent
Tree Nuts, Almond	Absent
Tree Nuts, Brazil	Absent
Tree Nuts, Cashew	Absent
Tree Nuts, Chestnut	Absent
Tree Nuts, Coconut and Oils	Absent
Tree Nuts, Hazelnut / Filbert	Absent
Tree Nuts, Hickory	Absent
Tree Nuts, Macadamia	Absent
Tree Nuts, Pine	Absent
Tree Nuts, Pistachio	Absent
Tree Nuts, Queensland	Absent
Tree Nuts, Walnut	Absent
Buckwheat	Absent
Bee Pollen/ Propolis	Absent
Royal Jelly	Absent
Pork	Absent
Peach	Absent
Tomato	Absent
Sulfur Dioxide and Sulfite > 10 ppm	Absent

Meaning of Present/Absent/Present-Exempt
Present - Intentionally added during the production process.
Absent - Not intentionally added during the production process.
Present-Exempt - Intentionally added during the production process, however have been given exemption from being required to be labeled on the final package.

The above list of allergens is in accordance with all applicable EU and US legal requirements.

Additional allergen information is available in the Product Information Sheet for
PROMITOR™ Soluble Corn Fiber 85A

GMO Status

For additional information regarding the GM status of the above-mentioned ingredient, please refer to the Product Information Sheet.

Storage Conditions

Container	Storage Temperature	Storage conditions to achieve maximum shelf life
Multiwall Bag		Store at ambient temperature and humidity.

Shelf Life

Package	Shelf Life	Sulfur Dioxide and Sulfite Level
Multiwall Bag	730 Days	< 10 ppm

Organic Statement

Certified for Organic Labeling: No

For more information please consult the Product Information Sheet for PROMITOR™ Soluble Corn Fiber 85A

Nutritional Information

Nutritional Information	Nutrients Per 100 Grams
Calories	114 kca
Calories from Fat	0
Total Fat	0 g
Saturated Fat	0 g
Trans Fat	0 g
Cholesterol	0 mg
Total Carbohydrate	95.0 g
Sugars	1.9 g
Dietary Fiber	80.8 g
Protein	0 g
Sodium	0 mg
Vitamin A	0.000 µg (0 IU)
Vitamin C	0 mg
Calcium	0 mg
Iron	0 mg
Moisture	5.0 g
Ash	0 g

Note: A value of 0 means this product is not a significant source of this nutrient.

United States Format

Supplier Information

Name:	Tate & Lyle
Address:	2200 E Eldorado Decatur, IL 62525 USA
Main Phone:	1-217-423-4411
Main Fax:	1-217-421-2628
E-mail:	orders@tateandlyle.com

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TATE & LYLE

Specification Sheet **300000001486**

STAR-DRI® 1015 A Maltodextrin

1630011115800, 1630011115021, 16300111

Appearance: Powder
Color: White
Odor:

Solubility Statement: Soluble In Water

Quality Inspection Plan (Official Specification Values)

Parameter	Target	Range/ Attribute	Unit	Method
MOISTURE		<= 6.00	%	Moisture - TN46020
pH		4.0 - 6.0		pH - TN60550
FOREIGN MATTER		<= 4	ppm	Foreign Matter - TN32545
DENSITY		15.00 - 18.00	lb/scf	Bulk Density - TN24551
LOOSE BULK DENSITY		240 - 289	g/dm^3	BULK DENSITY - TN24505
PACKED BULK DENSITY		288 - 385	g/dm^3	BULK DENSITY - TN24505
PACKED BULK DENSITY		18.0 - 24.0	lb/scf	BULK DENSITY - TN24505
FLOW RATE		5.0 - 10.0		Flow Rate - TN31340
SOLUBILITY		<= 30.0	s	Solubility - TN76000
DE		9.00 - 12.00	%	Dextrose Equivalent - TN25617
SCREEN THRU US#20		>= 97.00	%	Screen Size - TN69700
SCREEN ON US#100		>= 65.00	%	Screen Size - TN69700
SCREEN THRU US#200		<= 10.00	%	Screen Size - TN69700
BACTER TOTAL		<= 2000	n/g	Total Plate Count - TN10560
YEAST SPORE		<= 50	n/g	Mold & Yeast - TN47010
MOLD		<= 50	n/g	Mold & Yeast - TN47010
THERMOPHILES, TOT		<= 150	n/10g	Thermophilic Spores (NFPA) - TN16010
FLAT SOURS		<= 75	n/10g	Thermophilic Spores (NFPA) - TN16010
SULFIDE SPOR		<= 5	Mold/10gr	Thermophilic Spores (NFPA) - TN16010

GAS FORMERS		<= 4	per 6 tube	Thermophilic Spores (NFPA) - TN16010
SALMONELLA		Negative		SAL B/HW - TN10545
E. COLI		None Detected		Coliforms - TN10510
COLIFORMS		<= 10	n/g	Coliforms - TN10510

Product Composition / Labeling

Maltodextrin

Regulatory Status

Compliance Statement:

- Complies with FDA Regulation 21CFR184.1444 as Maltodextrin - generally recognized as safe.

21 CFR References: GRAS, 21CFR184.1444

Country of Origin

Country of Manufacture: United States

Kosher Status

Certified Kosher: Yes
Certified Kosher for Passover: No

The above-mentioned ingredient or its components is certified Kosher by:

- Orthodox Union (OU)

Halal Status

Certified Halal: Yes

Vegetarian Status

Vegetarian: Suitable
Vegan: Suitable
Ovo-Vegetarian: Suitable
Lacto-Vegetarian: Suitable
Lacto-Ovo-Vegetarian: Suitable

Presence of Allergens

If present in this Tate & Lyle ingredient, allergens will be denoted as “Present” in the table below.

Allergen	Status

Crustacean & Shellfish	Absent
Mollusk	Absent
Fish	Absent
Egg	Absent
Milk (including Lactose)	Absent
Peanut & Oils	Absent
Soybean & Oils	Absent
Gluten Containing: Wheat, Rye, Barley, Oats, Spelt, Kamut, Triticale	Absent
Seeds, Sesame & Oils	Absent
Celery/Celeriac	Absent
Mustard	Absent
Lupin	Absent
Tree Nuts, Almond	Absent
Tree Nuts, Brazil	Absent
Tree Nuts, Cashew	Absent
Tree Nuts, Chestnut	Absent
Tree Nuts, Coconut and Oils	Absent
Tree Nuts, Hazelnut / Filbert	Absent
Tree Nuts, Hickory	Absent
Tree Nuts, Macadamia	Absent
Tree Nuts, Pine	Absent
Tree Nuts, Pistachio	Absent
Tree Nuts, Queensland	Absent
Tree Nuts, Walnut	Absent
Buckwheat	Absent
Bee Pollen/ Propolis	Absent
Royal Jelly	Absent
Pork	Absent
Peach	Absent
Tomato	Absent
Sulfur Dioxide and Sulfite > 10 ppm	Absent

Meaning of Present/Absent/Present-Exempt
Present - Intentionally added during the production process.
Absent - Not intentionally added during the production process.
Present-Exempt - Intentionally added during the production process, however have been given exemption from being required to be labeled on the final package.

The above list of allergens is in accordance with all applicable EU and US legal requirements.

Additional allergen information is available in the Product Information Sheet for
STAR-DRI® 1015 A Maltodextrin

TATE & LYLE

GMO Status

The above-mentioned ingredient is not considered Non-GMO

For additional information regarding the GM status of the above-mentioned ingredient, please refer to the Product Information Sheet.

Storage Conditions

Container	Storage Temperature	Storage conditions to achieve maximum shelf life
Multiwall Bag		Cool dry environment.

Shelf Life

Package	Shelf Life	Sulfur Dioxide and Sulfite Level
Multiwall Bag	730 Days	< 10 ppm

Organic Statement

Certified for Organic Labeling: No

For more information please consult the Product Information Sheet for STAR-DRI® 1015 A Maltodextrin

Nutritional Information

Nutritional Information	Nutrients Per 100 Grams
Calories	378 kca
Calories from Fat	0
Total Fat	0 g
Saturated Fat	0 g
Trans Fat	0 g
Cholesterol	0 mg
Total Carbohydrate	94.5 g
Sugars	3.5 g
Dietary Fiber	0 g
Protein	0.30 g
Sodium	100.0 mg
Vitamin A	0.000 µg (0 IU)
Vitamin C	0 mg
Calcium	2.0 mg
Iron	0.5 mg
Moisture	5.0 g
Ash	0.4 g

Note: A value of 0 means this product is not a significant source of this nutrient.

Supplier Information

Name: Tate & Lyle
Address: 2200 E Eldorado
Decatur, IL 62525
USA
Main Phone: 1-217-423-4411
Main Fax: 1-217-421-2628
E-mail: orders@tateandlyle.com

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APPENDIX 2
Adverse Events Relatedness Reference Sheet (Cancer Consortium Clinical Research Support)

FRED HUTCH
UNIVERSITY OF WASHINGTON
CANCER CONSORTIUM

Clinical Research Support
Adverse Event Relatedness Reference Sheet

Relatedness (attribution) of adverse events to a study intervention must be assessed in accordance with the study protocol. In the event that a protocol does not provide assessment criteria, the descriptions below may be used.

Relatedness of Adverse Events to an Intervention	
Definite (must have all 4)	• Has a reasonable temporal relationship to the intervention • Is not readily associated with the subject's clinical state, concomitant medications/interventions, or other identifiable factors • Follows a known pattern of response related to the specific intervention • Disappears or decreases with reduction in dose or cessation of intervention and recurs with re-exposure (if applicable)
Probable (must have 3)	• Has a reasonable temporal relationship to the intervention • Is not readily associated with the subject's clinical state, concomitant medications/interventions, or other identifiable factors • Follows a known pattern of response to intervention • Disappears or decreases with reduction in dose or cessation of intervention
Possible (must have 2)	• Has a reasonable temporal relationship to the intervention • Has unknown association with the subject's clinical state, concomitant medications/interventions, or other identifiable factors • Follows a known pattern of response to intervention
Unlikely (must have 2)	• Does not have a temporal relationship to the intervention • May be associated with the subject's clinical state, concomitant medications/interventions, or other identifiable factors • Does not follow a known pattern of response to intervention • Does not change with reduction in dose or cessation of intervention, and/or does not reappear or worsen with reintroduction of intervention (if applicable)
Unrelated (must have all 4)	• Does not have a temporal relationship to the intervention • Is associated with the subject's clinical state, concomitant medications/interventions, or other identifiable factors • Does not follow a known pattern of response to intervention • Does not change with reduction in dose or cessation of intervention, and/or does not reappear or worsen with reintroduction of intervention (if applicable)

This content is based in part on the National Institute of Allergy and Infectious Disease (NIAID) definitions of relatedness to adverse events.