The Role of Targeted Prebiotics for Improving Gut Microbiome Composition and Function in Individuals Consuming Diets Low in Fiber, and Individuals at Risk for Chronic Disease

By

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Abstract

Diet is known to be a strong determinant of gut microbiome composition and function, and it is also known that gut microbiome dysbiosis is strongly linked to the risk of chronic disease. However, it is not clear how to intervene through diet or supplementation to beneficially alter the gut microbiome in individuals who have dysbiosis or who are at risk for chronic diseases such as cardiovascular disease and Alzheimer's disease (AD). Hypothesis driven studies to determine how dietary changes affect the diet-gut microbiome-host interrelationship are critical for the discovery of solutions to improve health and mitigate disease risk. In this dissertation, three chapters are included to discuss important aspects of study design and prebiotic selection, and the outcomes of a study investigating the effects of a prebiotic supplement.

In the first chapter, critical factors in successful study design for diet-microbiome research are discussed. For example, the importance of detailed dietary data collection and analysis, tight control of background diet, and stabilizing diet in the days prior to sample collection is critical for reducing noise and amplifying the signal that can be detected of the effects of the intervention diet. Specific recommendations such as including multiple consecutive sampling per study timepoint and collection of multiple consecutive days of diet records, particularly the days prior to sample collection, are discussed. High interindividual variability in gut microbiome composition is a well-established phenomenon that has been known since the early results from the Human Microbiome Project. Because of this high interindividual variability, some study designs are not well suited to discovering the effects of a dietary intervention. Instead, we discuss the double-blinded, randomized order, placebo-

controlled, cross-over study design is the optimal study design for gut microbiome research, enabling the detection of the effects of even a subtle diet intervention. We also discuss the importance of precision nutrition approaches for nutritional recommendations. As an example, we discuss nutritional recommendations for egg intake, taking into account the link between egg-derived components (i.e., choline and cholesterol) and their interaction with the gut microbiome and subsequent health outcome measures important in cardiovascular disease.

The second chapter emphasizes why we care about changes in gut microbiome in human health and how we can better utilize certain dietary interventions such as prebiotics to modulate human health and diseases focusing particularly on AD. Prebiotics are oligosaccharide and polysaccharide structures isolated from plants or synthesized from sugars that are shown to modulate the microbiome and are associated with health benefits. We summarized the importance of dietary fiber intake for maintaining a healthy gut microbiota, with a focus on short chain fatty acid production, mucus secretion, and protection from pathogens. The specific links between healthy gut physiology, gut immune function and the brain are also discussed. The importance of anti-inflammatory metabolites produced by gut microbes consuming specific substrates in modulating critical signaling pathways in the brain is discussed in the context of AD prevention. We conclude by discussing which specific bacterial genera are increased and decreased in AD patients and highlight the potential of increasing bifidobacteria abundance through the intake of specific prebiotic substrates as a promising approach for beneficially modulating the gut microbiome and improving health outcomes in AD patients.

The final chapter summarizes the findings from our human clinical trial in healthy subjects consuming a low fiber diet (< 15 g/day) to determine whether a low-dose, easy to use

bifidogenic prebiotic supplement formulation increases bifidobacteria and produces measurable metabolomic and metagenomic changes over the course of 4 weeks. We used the double-blinded, placebo-controlled, randomized order crossover study design highlighted as the optimal study design for diet-microbiome studies in chapter 1, to study the effects of the prebiotic supplements in 20 otherwise healthy young individuals consuming diets low in fiber, typical of the average American diet. As hypothesized, the relative abundance of bifidobacteria significantly increased after the prebiotic supplement, as did the gene counts of genes associated with prebiotic utilization, acetate production, and choline oxidation. Importantly, these changes in gut microbiome composition and function were reflected by changes in the concentrations of microbially-derived metabolites in the plasma, and point to novel pathways by which increasing the abundance of bifidobacteria may confer beneficial health effects on the host.

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Chapter 1.1. The Optimal Gut Microbiome Study Design and Fecal Collection Methods

1. Introduction

High interindividual variability in dietary intake and microbiome composition are important factors that complicate our understanding of the effects of diet on the gut microbiome. Variables that affect the study of relationships between diet and gut microbial communities in humans, emphasizing the importance of choosing the optimal study design, are discussed. Practical suggestions for fecal sample collection, transport, and storage to minimize variability and improve the signal to noise ratio are also discussed.

2. Optimal Study Design for Gut Microbiome Study

To understand the impact of dietary components on the gut microbiome study design is a key factor to reduce the high interindividual variation in both diet and gut microbiome composition. The key advantage of implementing the cross-over study design in diet-gut microbiome intervention studies is that each individual acts as their own control allowing researchers to conduct a within-person comparison. Studies implementing the cross-over study design have successfully demonstrated the ability to reduce the confounding factor of interindividual variability (1–3). In addition to the cross-over design, the randomization of intervention order, a placebo control intervention arm, and double-blinding of both study personnel and participants are factors to include and consider in an overall study design to minimize practical errors in the study protocol.

Additional important factors to consider in the study of the effects of dietary interventions and supplements on gut microbiome are habitual diet and well-defined

inclusion/exclusion criteria. In such studies, the willingness of study participants to adhere to all required study protocols during the study period is crucial. It is critical to control for and account for medical history or lifestyle changes during the study. Tracking adherence to and compliance with study protocols is a primary responsibility of well-trained study personnel. The use of validated questionnaires such as health history, 3-day diet records, the Bristol stool scale, bowel movement and frequency, and physical activity are important tools in monitoring both compliance and effects of the intervention (4,5).

3. Strategies for Fecal Sample Collection

In addition to the factors related to study design and execution discussed above, successful microbiome studies must consider strategies for fecal sample collection, transport, and storage, given that the abundance and composition of live microorganisms in the collected sample may fluctuate from the collected time point and thus influence results. Comparisons of several of the collection methods have been detailed in several recent publications (6–9). The most crucial parameter is the collection method which then also dictates the need for control of temperature and transport time.

Many fecal collection kits and collection strategies are available, and the principal advantages and disadvantages are briefly reviewed here. The kit selection depends on the amount and type of sample needed for downstream analyses, as well as the conditions and available resources associated with the study design. Swab-based fecal collection is a way for subjects to easily obtain specimen from the toilet paper or collected stool. The American Gut Project sampling methodology using a swab to take a small amount of fecal material from a

stool or soiled toilet paper did not significantly alter microbial diversity and composition as measured by 16S sequencing compared to other methods (10). However, swab-based collection is not ideal for studies where larger amounts of fecal sample are needed for multiple analyses, for example metabolomics in addition to sequencing (11).

The alternatives to swabs are collection of the entire bowel movement or collection of a subsample of the bowel movement into tubes or containers. The collection of the entire bowel movement may be ideal considering the non-homogenous distribution of gut microbiota in a single bowel movement (12,13). Fecal sampling location may affect the abundance of specific microbes at the family level reflecting the diverse microbial communities residing in small niches throughout the gastrointestinal tract (14). Subsampling may thus particularly impact the recovery of low abundance taxa and over- or under-represent the relative abundance of certain taxa (15). On the other hand, although subsampling from different sections of stool resulted in small changes in gut microbial composition, the differences were relatively minor compared to inter-individual variability (16). Collection of the entire bowel movement presents significant logistical challenges including the need for study participants to collect and transport large sample volumes, and for study personnel to allocate large amounts of freezer space and time to homogenize and process large sample volumes. Given these significant challenges, the collection of the entire bowel movement is not a common sampling strategy that is used in clinical studies.

Subsampling can be a random selection of any part of the collected bowel movement, or a certain number of distinct sections of the bowel movement (e.g., three samples, one from the beginning, one from the middle, and one from the end). Subsampling of the bowel

movement is typically performed by the study participant using gloves and sterile spatulas. The participant can transfer the sample into small tubes such as Eppendorf tubes, which can be immediately stored at −80°C in regular freezer boxes, minimizing sample handling and storage space needs, as well as diminishing DNA degradation from freeze-thaw cycles (17). Alternatively, the participant can collect sample into larger stool collection containers, which offer the advantage of a larger sample volume but require aliquoting into smaller tubes that are amenable to long-term storage in the −80°C when received into the lab, or repeated freeze-thaw cycles to aliquot as needed and more space needed in the freezer.

Regardless of which sampling and collection technique is chosen, the collection container can additionally be prefilled with preservative, which allows sample to be shipped and stored at ambient temperature for 7 days (18) or as long as 60 days depending on the preservative used (19). The preservative filled tubes are ideal for situations where participants are remote from the site of the research study, or if refrigeration between collection and storage at −80°C is not possible. However, preservative-filled tubes may interfere with some downstream analyses and experiments, for example metabolomics (20) and culturing. If tubes without preservative are used, samples need to be temperature controlled from the point of collection (e.g., placed in coolers with ice packs or dry ice, temporary storage in home freezers) until arrival to the −80°C freezer in the research facility, in order to prevent DNA degradation and bacterial growth. Immediate sample storage at −80°C is considered the gold standard regardless of the presence of preservative.

4. Conclusions

In a human clinical study, planning for the optimal study design based on the study aims is crucial. Human clinical studies, which aim to analyze samples that are high in interindividual variation, should consider a cross-over study design. Additionally, randomization of intervention order, placebo-controlled, and double-blinded design increases the reliability of the data. Importantly, managing the study protocol and follow-up on compliance of study participants are key factors to success for clinical studies aiming to assess the effects of diets on gut microbiome composition. The use of validated questionnaires is an important tool for keeping track of background diet, sample collection, health status, and adherence to the study diet or supplement during the study period.

Strategies for proper fecal sample collection, transport, and storage is also critical. Researchers should choose the optimal collection method based on their sample analysis plans (e.g., DNA extraction and sequencing, metabolomics, proteomics, etc.). Lastly, the transport and storage of fecal samples should be as quick as possible using temperature-controlled containers with storage at −80°C.

Chapter 1.2. Are Eggs Good Again? A Precision Nutrition Perspective on the Effects of Eggs on Cardiovascular Risk, Taking into Account Plasma Lipid Profiles and TMAO

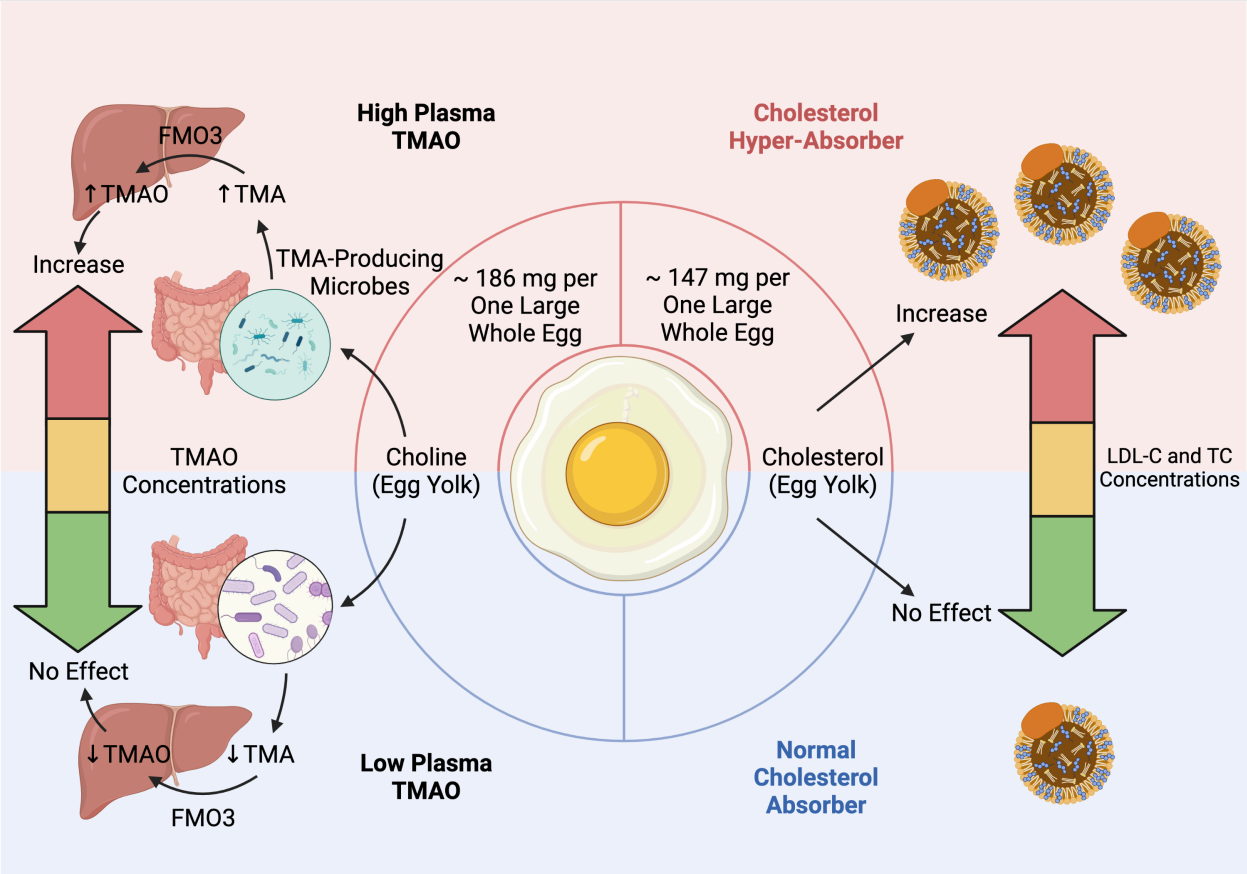


Figure 1. Graphical abstract on the mini review.

1. Introduction

Eggs are a complete and convenient food packed with macro- and micronutrients, providing 7 g of complete protein, minerals such as Zn, bioactive antioxidant and anti-inflammatory components such as lutein and zeaxanthin, phospholipids, and a host of other nutrients, per 80-Calorie egg (21). However, eggs are also enriched with cholesterol and choline, which have the potential to have deleterious rather than beneficial effects on cardiovascular disease (CVD) risk in certain population groups. In order to make Precision

Health-based dietary recommendations to individuals it is critical to examine the appropriateness of any individual food or dietary component in the context of the risk and benefit profile of consuming that food at the individual level. In this mini review we evaluate recent evidence from dietary intervention studies on the effects of egg intake on cardiometabolic risk factors, including plasma lipid profiles and the novel gut microbiome-derived metabolite trimethylamine-N-oxide (TMAO). We also outline a strategy to clinically evaluate the appropriateness of egg intake for individuals at risk for CVD.

2. Do Eggs Raise Plasma Cholesterol?

Dietary cholesterol intake was at first thought to be associated with increasing plasma cholesterol concentrations (22), but it was later discovered that in most cases dietary cholesterol does not in fact raise plasma cholesterol, except in individuals with genetic polymorphisms in the ABCG5 and ABCG8 genes, which result in cholesterol hyperabsorption at the level of the intestinal tract (23). Gut bacteria may also play a role in cholesterol absorption as individuals with microbes forming coprostanol have lower levels of fecal and serum cholesterol (24). Interestingly, the decision has been made to remove the dietary cholesterol restriction of 300 mg/d from the US Dietary Guidelines (25), given recent evidence that dietary cholesterol, when not accompanied by high saturated fat content as with eggs, does not tend to raise plasma cholesterol in most individuals in both observational and intervention trials (26), and recent meta-analyses of large prospective US cohort studies showing that consumption of 1 egg per day is not associated with overall CVD risk factors (27).

The assumption of course, is that the dietary guidelines are meant for the "average" American and not individuals who may have the underlying genetic predisposition to hyper-absorb cholesterol. Because one egg contains approximately 200 mg cholesterol, it is important to understand whether for a given individual egg intake is appropriate due to low/normal dietary cholesterol absorption or inappropriate because of underlying genetic factors that increase cholesterol absorption and thus raise plasma cholesterol. Hyper-responders and hypo-responders to dietary cholesterol are classified as individuals whose total cholesterol concentrations increase (>2.2 mg/dL) or decrease (< 2.0 mg/dL) for every 100 mg/d of dietary cholesterol consumed, respectively (28). The prevalence of sitosterolemia is at least 1 in 2.6 million and 1 in 360,000 for the *ABCG5* and *ABCG8* gene mutations, respectively, suggesting that a very small percentage of the population is affected by the known mutations (29). There are tests available to clinicians that measure markers of cholesterol absorption (concentrations of phytosterols, campesterol and sitosterol) (30) that may be useful in helping to identify individuals who are likely to be responders to dietary cholesterol, and for whom eggs and other sources of dietary cholesterol should be minimized to reduce plasma cholesterol and CVD risk. For the remainder of individuals, it appears that egg intake is not associated with deleterious changes in lipoprotein profiles. In fact, recent studies have shown that although consuming 2-3 whole eggs per day did not change total cholesterol or LDL-cholesterol concentrations, it did increase paraoxonase-1 (PON1) activity, in healthy individuals (31) and the cholesterol efflux capacity of HDL particles in overweight postmenopausal women (32), as well as in individuals with metabolic syndrome who were consuming eggs in the context of carbohydrate restriction

(33), suggesting that in some individuals there may even be a beneficial effect of egg intake on CVD risk profiles.

Thus, whether eggs raise plasma cholesterol depends on the individual. In most cases, dietary cholesterol intake in the form of eggs, since it is not accompanied also by an increased saturated fat intake as would be the case with meat, does not raise plasma LDL or total cholesterol, and may even improve HDL cholesterol efflux capacity. In a clinical practice where patients at risk for CVD or with a family history of CVD are being treated, and where there is therefore a higher likelihood of finding individuals who may have one of the ABCG5 or ABCG8 mutations that contribute to cholesterol hyperabsorption in the intestinal tract, it may be beneficial to do further testing, such as measurement of plasma phytosterol concentrations to determine whether egg intake is appropriate for the individual patient.

3. Do Eggs Raise Plasma TMAO?

Fasting plasma concentration of TMAO is a risk factor for CVD even after adjustment for other CVD risk factors (34). However, whereas there is a clear positive association between fasting plasma TMAO concentrations and CVD risk (35), and increased production of TMAO in many individuals in the hours immediately following the intake of eggs (34,36), studies have not shown a consistent link between the dietary intake of eggs and long-term fasting TMAO concentrations. Controlled intervention studies feeding 2-3 eggs per day have not shown increases in TMAO concentrations (37,38). In comparison, observational studies show links between higher intakes of fish, and less so eggs, and circulating TMAO concentrations (39). Thus, it is likely that the production of TMAO related to egg intake is highly individual, and

dependent on a number of factors, including the individual's gut microbiome profile. It is important to note that since the conversion of choline to TMA by gut microbes is the first step, while the further conversion of the absorbed TMA to TMAO in the liver is the second step in the synthesis of circulating TMAO, the rate of TMAO production is also affected by the individual patient's liver flavin-containing monooxygenase 3 (FMO3) activity (40). Several factors are known to modulate FMO3 expression and activity including estradiol (41), and bile acids (40) via the farnesoid x receptor (40). Moreover, TMAO can also be absorbed directly from dietary sources particularly fish (39), and its concentrations are also dependent on the rate of clearance from plasma and thus kidney function (42).

Egg lipids as a source of choline in the form of phosphatidylcholine have been shown to result in increased concentrations of TMAO in the hours after consumption of 2 or more eggs (36). Sphingomyelin is an additional source of choline from eggs, however, dietary sphingomyelin has been shown to have only modest effects on increasing circulating TMAO concentrations in mice (43). There is high inter-individual variability in TMAO production following egg consumption, with the observation that individuals who follow a vegan diet do not produce TMAO whereas omnivores do (36). Strikingly, although vegans do not produce TMAO at all after ingesting eggs and other sources of choline, omnivores are highly variable in their TMAO production ranging from near 0 μM, just as in the vegans, to as high as 30 μM (36). It has now been shown that variation in the gut microbiome contributes to differences in TMAO production (44) and the gut microbes involved in this relationship include those belonging to the phyla *Firmicutes* and *Proteobacteria* (45,46), however the relationship between diet, the gut microbiome, and circulating TMAO concentrations is complex.

Although it is now clear that shifts in gut microbiome composition can be seen as quickly as within 4 days of a major shift in diet (e.g. from a low-fiber, high fat "fast food" diet of burgers and fries, to a high-fiber Mediterranean diet), TMAO concentrations did not change at this time scale (47). On the other hand, in a crossover intervention study between a plant-based (Plant) and animal-based (Animal) diet for 8 weeks each in healthy adults, in the group randomized to consuming the Animal diet first and Plant diet second, TMAO concentrations decreased without significant changes in gut microbiome composition (48). It was recently shown that fecal microbiota transplantation from vegan donors to patients with metabolic syndrome changed the composition of the gut microbiome, however, these changes were not associated with reduced TMAO concentrations, and in fact, the relative proportion of TMA-generating species *Alcaligenes faecalis* increased after the fecal microbiota transplantation (49). Whether a particular bacterial species within a family, genus, or phylum produces TMA from choline or other sources like carnitine, is dependent on whether that species carries the metabolic machinery required for the conversion steps. Metagenomic sequencing combined with bioinformatic approaches have recently revealed that the microbial enzymes necessary for the conversion of choline and carnitine to TMA include the *cut* gene cluster, which has been identified in 89 bacterial genomes including known choline degraders (*Desulfovibrio*, *Clostridia*, *Streptococcus*, *Klebsiella*, and *Proteus*) (50,51).

In addition to the importance of the composition of the gut microbiome, including specifically whether the microbes present in the gut have the necessary genetic machinery to convert choline and carnitine to TMA, the availability of the substrate to the microbes is also a factor. Since egg choline is present mostly in the form of phosphatidylcholine, which is known

to be readily absorbed in the upper gastrointestinal tract (52), there may be less choline available to the gut microbes, which are concentrated in the lower intestinal tract, than when choline is consumed in other forms. Sources of free choline such as choline bitartrate may be more easily accessible to gut microbes because their absorption is not as efficient as that of choline in the phosphatidylcholine form (53). These differences in the absorption of free choline vs. choline in the form of phosphatidylcholine may affect its metabolism by gut microbes, and thus also the rate of TMAO production and appearance in the bloodstream. When choline (600 mg) in the form of choline bitartrate was given to healthy men it induced higher plasma and urinary TMAO changes from baseline compared to equal amounts of choline in the form of phosphatidylcholine (54). In the same study it was found that choline bitartrate feeding resulted in increased concentrations of *Clostridium* from *Ruminococcaceae* and *Lachnospiraceae* in the phylum *Firmicutes* in high-TMAO producers compared to low-TMAO producers (54). These observations are in agreement with a prospective cohort study showing an association between fecal levels of *Ruminococcaceae* and *Lachnospiraceae* and plasma TMAO concentrations (55).

Thus, the gut microbiome is an important determinant of whether an individual's TMAO concentrations are likely to increase in response to egg intake. Many companies and laboratories now offer fast, and ever less expensive metagenomic sequencing services, and machine learning approaches are being widely deployed to develop algorithms to process the sequencing data. However, the evaluation of an individual's gut microbiome composition to predict the extent of TMA production in response to choline intake is currently still in the research realm and has not yet been translated into a clinical diagnostic. Instead, the direct

measurement of TMAO in plasma is an available approach to evaluate an individual patient's TMAO status. Some states have already approved the measurement of TMAO in plasma for prognostic purposes, and some clinical labs already offer routine TMAO analysis for clinicians who want to order this test for their patients. In individuals who already have high TMAO concentrations, it would be inappropriate to recommend increases in the intake of choline and carnitine, and in fact, decreased intakes of food sources, and especially supplements, of these molecules would be prudent. On the other hand, in individuals for whom eggs are already an important and regular source of protein and other nutrients in the diet, and whose TMAO concentrations are low, it would be beneficial to recommend keeping eggs in their diet. It is important to consider an individual's overall diet and to keep track of total protein intake and protein source. In the case of an individual in whom plasma TMAO concentrations are high, if the overall diet includes high amounts of choline, carnitine, or TMA-containing foods (i.e., meats and fish) and these foods are the primary sources of protein, it may take some time, effort, and education to transition to alternative protein sources. Based on the current evidence, which is primarily from observational studies, a focus on reducing meat (particularly processed meat products) and fish rather than eggs would be a reasonable approach for reducing plasma TMAO concentrations. Interventional studies using precision nutrition-based approaches are needed to better understand how substituting choline, carnitine, and TMA-containing foods with specific protein alternatives affects the gut microbiome and plasma TMAO concentrations in individuals.

It is important to note that most of the studies on egg and choline intake and TMAO production have been performed in healthy individuals with normal gastrointestinal function,

thus it is important to consider that individuals with decreased absorption capacity due to a number of possible underlying lipid malabsorption conditions including disorders of bile acid metabolism, liver, pancreatic and gall bladder disease, inflammatory bowel disease, and other gastrointestinal disorders may in fact produce higher levels of TMAO in response to egg consumption due to a failure to absorb the phosphatidylcholine in the upper gastrointestinal tract, and thus greater availability of this precursor to TMA-producing gut microbes in the colon.

4. Conclusions

In this mini review, we evaluated the current evidence on the effects of egg intake on cardiometabolic risk factors including plasma cholesterol and TMAO levels, with a specific focus on individuals at risk for CVD. In those patients at risk for CVD, whose plasma cholesterol concentrations are unaffected by dietary cholesterol intake, and whose diet and microbiome are not associated with increased TMAO concentrations, eggs are beneficial to incorporate or keep in the diet. This has not been evaluated in clinical studies directly, thus it is critical that clinicians carefully evaluate the individual needs of their patients based on their individual status and risk. Some clinical tools, including the measurement of phytosterols to assess the extent of dietary cholesterol absorption, and the measurement of TMAO to assess the extent of TMAO production in the context of diet, are already available. It is likely that these measures are more informative and can better aid the clinician in determining the appropriateness of egg intake in the context of careful evaluation of the patient's diet in the weeks prior to the blood draw, since both the microbiome and plasma metabolites change depending on recent diet

(31,32,37,38,47,48). Given that eggs are a convenient and excellent source of both macro- and micronutrients, they may be an important dietary tool to achieve adequate nutrient status in some individuals, even those at risk for CVD. However, many factors including personal genetic background, absorption and bioavailability differences, the integrity of the patient's gastrointestinal tract and associated digestive organs, and the individual's microbiome should all be considered in patients at risk for CVD to prevent high plasma cholesterol and TMAO levels due to egg intake. Further studies are needed to develop Precision Nutrition approaches that can identify patients at risk for CVD for whom egg intake is contraindicated and others for whom egg intake is not associated with increased plasma cholesterol or TMAO.

Chapter 2. The Potential Utility of Prebiotics to Modulate Alzheimer's Disease: A Review of the Evidence

1. Introduction

Microbiota dysbiosis, characterized as the disproportional increase or decrease in abundance of certain bacterial strains, has been associated with multiple complications, including obesity (56), type 2 diabetes (T2DM) (57), and neurodegenerative diseases such as Alzheimer's Disease (AD) (58). AD is the most common neurodegenerative disease affecting about 5 million people in the U.S., and about 25 million people worldwide (59). Only about 5–10% of AD patients present with early onset dementia directly linked to genetic mutations that are causal for AD development (60). The vast majority of AD patients, on the other hand, develop neurodegenerative disease due to a combination of factors including but not limited to apolipoprotein E genotype (61,62), presence of metabolic syndrome and certain lifestyle factors (63), and, as recently revealed, microbiome composition (58). AD is a neurodegenerative disease characterized by memory loss and a progressive loss of cognitive function involving the extracellular accumulation of pathogenic amyloid-β (Aβ) peptides that oligomerize and aggregate, forming plaques (64), and the intracellular accumulation of hyperphosphorylated tau proteins that form neurofibrillary tangles (65). The causes for the formation of Aβ plaques and neurofibrillary tangles are not clear. However, chronic neuroinflammation and dysfunctional microglia have emerged as key drivers of these processes (66,67). Notably, neuroinflammation has recently been found to be modulated by the gut microbiome via the gut-brain axis (68). The links between microbiome composition and AD are intriguing and provide potential ways to ameliorate or even prevent AD progression through modifying the

microbiome. This could be achieved via various ways, including fecal transplant and consumption of probiotics or prebiotics. Prebiotics are oligosaccharide molecules that are non-digestible to the human host, and which serve as substrates for microorganisms in the gut, and thus modulate the composition and/or function of gut microbes in a manner that is beneficial to the host (69,70). In this review, we discuss the evidence linking gut microbiome composition and function with AD and its associated co-morbidities, provide an overview of prebiotic types and their effects, discuss evidence for the effectiveness of prebiotics in modulating gut microbiome composition and microbial metabolite production, and discuss the potential for prebiotics to induce a beneficial shift in the gut microbiome and modify health outcomes relevant for individuals with AD.

2. Links between Gut Microbiome Composition and AD and Associated Co-Morbidities

The importance of diet in modifying the gut microbiome has been emphasized through many intervention studies in humans and animal models. Studies have demonstrated that diet affects gut microbiota composition and diversity (71–80). Diet composition and duration of intervention are the two most relevant diet-related factors in shaping the gut microbiome. The most well-studied dietary interventions thus far have involved the comparison of high-fat or Western diets enriched in animal-derived foods vs. lower-fat or plant-based diets (Figure 2). From animal studies to human studies the diversity and proportion of microbes have been found to be consistently altered by diets depleted vs. enriched in plant substrate. Specifically, diets depleted in non-digestible fiber and enriched in protein and fat have been consistently linked with an increase in protein- and fat-degrading bacteria belonging to the phyla Firmicutes,

Proteobacteria, and Deferribacteres, and a decrease in Bacteroidetes and butyrate-producing species, which are generally known to be beneficial for human health (81–85). Conversely, fiber-enriched diets are typically associated with increases in the abundance of species in the phylum Bacteroidetes, the genus *Prevotella*, and *Bifidobacterium* spp. (86–90). These changes in gut microbiota composition are closely associated with host health and disease. The health effect is not only attributed to the enrichment of beneficial gut microbes but to the production of secondary metabolites such as short chain fatty acids (SCFAs) from the degradation of non-digestible carbohydrates by specific fiber-fermenting taxa (91–93). The presence of these taxa is associated with protection from AD, and a number of associated co-morbidities including T2DM and cardiovascular disease (CVD). In the next several paragraphs we review the evidence linking gut microbiome alterations to AD, as well as associated co-morbidities.

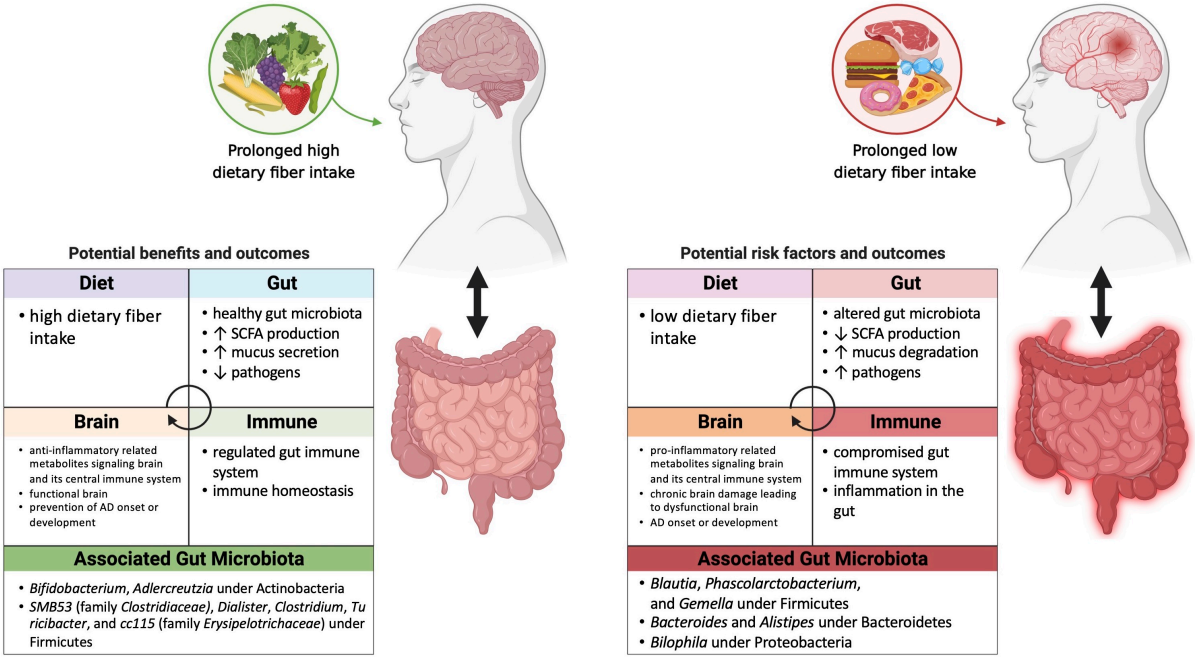


Figure 2. The potential association of prebiotic-gut-Alzheimer's Disease (AD) in individuals with prolonged high vs. low fiber diet.

The intake of dietary fiber may further influence gut health, immune system, and brain function. High dietary fiber intake may help maintain healthy gut microbiota, which is associated with increase in SCFA production, mucus secretion and decrease in pathogens. Healthy gut physiology leads to regulated gut immune system and immune homeostasis in which positively affects the brain. Anti-inflammatory metabolites signal the brain and its central immune system, which may potentially contribute to functional brain and prevention of AD onset or development. Bacterial genera that are shown to be less abundant in AD patients were *Bifidobacterium* and *Adlercreutzia* under Actinobacteria, *SMB53* (family *Clostridiaceae)*, *Dialister*, *Clostridium*, *Turicibacter*, and *cc115* (family *Erysipelotrichaceae*) under Firmicutes. Low dietary fiber intake may alter gut microbiota leading to dysbiosis in the gut, decrease in SCFA production, and increase in pathogens. Dysbiosis in the gut may cause compromised gut immune system and inflammation in the gut. Pro-inflammatory metabolites signal the brain and its central immune system and potentially bring chronic damage to the brain, which may result in dysfunctional brain, AD onset or development. Bacterial genera that are shown to be more abundant in AD patients were *Blautia*, *Phascolarctobacterium*, and *Gemella* under Firmicutes, *Bacteroides* and *Alistipes* under Bacteroidetes, *Bilophila* under Proteobacteria.

Studies have shown a connection between the composition and diversity of gut microbes and AD (Figure 2) (58,94,95). In a recent study a reduction in overall gut microbiome richness as well as decreases in *Bifidobacterium* and *Adlercreutzia* under Actinobacteria, *SMB53* (family *Clostridiaceae)*, *Dialister*, *Clostridium*, *Turicibacter*, and *cc115* (family *Erysipelotrichaceae*) under Firmicutes were observed in AD participants (58). On the other hand, *Blautia*, *Phascolarctobacterium*, and *Gemella* under Firmicutes, *Bacteroides* and *Alistipes* under Bacteroidetes, and *Bilophila* under Proteobacteria were increased in AD patients (58). In addition, 13 genera were associated with cerebrospinal fluid (CSF) biomarkers of AD (58), showing that gut microbiome composition or diversity may contribute to AD development. Firmicutes and Bacteroidetes are two dominant phyla in the human gut (96) and it has been observed that the Firmicutes/Bacteroidetes ratio is associated with obesity, gut dysbiosis, and a number of diseases including diabetes and CVD. However, the use of this ratio as an assessment

of the health state of the gut microbiota is controversial, as contradictory results have been reported (58,94,97–100). Gut microbiota composition assessment metrics that are based on measurements at the phylum level are unlikely to be useful since individual genera and species, even strains, under a particular phylum can play opposite roles in overall gut health, taking on different metabolic roles, producing different metabolites, and interacting with other microbes in the gut in different ways such that the overall effect of all individual species of that phylum is complex (Figure 2).

The onset and progression of AD has been linked directly to neurodegenerative processes secondary to the deposition of Aβ plaques and aggregation of hyperphosphorylated tau tangles (101). Recently, the pathogenesis of AD has been hypothesized further to be triggered by amyloid fibers of bacterial origin, which induce a proinflammatory response (102). A recent study found that amyloid-positive cognitively impaired patients had higher *Escherichia*/*Shigella* and lower *Eubacterium rectale* and *Bacteroides fragilis* abundances compared to amyloid-negative cognitively normal controls, and these compositional changes were correlated with an increased production of pro-inflammatory cytokines and a reduction of anti-inflammatory cytokines (103). In a cross-sectional study in Australian women consumption of a "junk food" (high sugar, high fat) diet was highly associated with Aβ deposition, whereas consumption of the Mediterranean diet was associated with higher cognitive scores than other diet groups (104). Interestingly, in a small study participants with mild cognitive impairment consuming a modified Mediterranean-ketogenic diet consisting of less than 20 g/d of carbohydrate were found to have higher abundances of *Enterobacteriaceae*, *Akkermansia*, *Slackia*, *Christensenellaceae* and *Erysipelotrichaceae* and lower abundances of saccharolytic

Bifidobacterium and *Lachnobacterium* compared to cognitively normal participants (105). In a follow-up study the low-carbohydrate modified Mediterranean-ketogenic was found to have a potential beneficial effect in AD patients in preventing memory decline (106). However, these studies were conducted in small cohorts (e.g., 17 individuals, 11 MCI patients and 6 controls), thus the effects of low-carbohydrate, low-fiber diets, even in the context of high monounsaturated and polyunsaturated vs. saturated fat ratios such as those seen in the Mediterranean diet, need to be further investigated in larger trials.

T2DM and AD have been known to share several pathophysiological features including hyperglycemia leading to increased Aβ production, and impaired glucose transport and subsequent glucose metabolism (107). A new potential AD biomarker, S100B, has been investigated to learn the common pathophysiology of these diseases (108). A cross-sectional study conducted with 100 South Indian AD patients showed that elevated levels of S100B protein in serum were significantly associated with clinical dementia rating scores compared to healthy controls (109). Serum S100B protein levels in T2DM patients were also shown to be positively correlated with cognitive function (110). In patients with clinically diagnosed T2DM a high-fiber diet composed of whole grains and prebiotics promoted strain specific growth of acetate and butyrate producing bacteria *Faecalibacterium prausnitzii*, Lachnospiraceae bacterium, and *Bifidobacterium pseudocatenulatum* (111). The treatment group had improved levels of hemoglobin A1c, as well as increased glucagon-like peptide-1 production compared to the control group (111). These results suggest the high-fiber diet induced gut microbial alteration is correlated with improvement of blood glucose regulation in T2DM patients. These

findings have important implications for the management of AD due to the high rates of T2DM comorbidity in AD patients.

In addition to a link with T2DM, CVD has also been linked with AD (112,113). The occlusion of blood vessels that support the deep brain result in silent brain infarcts (114). This type of infarct is shown to be associated with lower cognitive function related to attention, memory, and language (115). CVD may directly affect poor blood flow to the brain causing cerebrovascular disease (116). Meta-analyses of prospective cohort studies exploring the association of coronary heart disease with dementia or cognitive impairment found that coronary heart disease is associated with an increased risk of dementia or cognitive impairment (117,118). It is well-established that as much as 80% of the risk for CVD is attributable to diet and lifestyle factors (119–121). Many human studies have demonstrated an inverse association between the consumption of dietary fiber and the incidence of CVD (122–126). Patients with primary hypertension showed a high frequency of opportunistic pathogens such as *Klebsiella* spp., *Streptococcus* spp., and *Parabacteroides merdae*, whereas *Roseburia* spp. and *F. prausnitzii* which are SCFA-producers were abundant in healthy individuals (127). Another study found that total and LDL-cholesterol levels were lowered after the consumption of flaxseed fiber (128). However, although the consumption of maize-derived whole grain cereal led to increases in bifidobacteria, no significant changes were observed in serum lipids (129). Further studies examining the role of dietary fiber and specific increases or decreases of gut microbes as well as their metabolites on CVD endpoints are needed.

Taken together, the overall findings from the published literature suggest that modifying gut microbial composition and diversity toward a profile associated with healthy individuals

consuming healthy diets may help attenuate AD progression. Diets and prebiotic approaches that aim to increase beneficial bacterial species that have been found to be depleted in AD patients such as *Bifidobacterium* spp., and approaches that aim to decrease the abundance of deleterious bacterial species such as *Bilophila* may be beneficial for the prevention of AD (Figure 2).

3. Overview of Prebiotic Types and Their Roles in Modifying Gut Microbiota

Dietary fibers, which are somewhat difficult to define, can be classified according to their solubility. Insoluble fiber, which does not dissolve in water, passes through the digestive tract providing bulking by absorbing water. Soluble fiber, on the other hand, dissolves in water and is mostly fermented by commensal bacteria residing in the colon and contributing to satiety (130,131). Although this general categorization of fibers according to their solubility may be useful, insoluble fibers are fermented to a certain degree and some soluble fibers may be non-viscous. Recently, the classification of fiber according to functionality is gaining attention. The functionality depends on the structure and fermentability of the specific dietary fiber. Thus, types of dietary fiber and subsequent gut microbial composition, diversity, and richness changes are highly intriguing areas for further research. It is especially relevant to patients with AD given that particular dietary fibers may modify the gut microbiome in a beneficial direction, increasing the levels of metabolites that improve cognitive function and attenuate neurotoxicity (132). Here, we have listed a number of dietary fibers with known impacts on enrichment of certain gut microbes, suggesting their potential as prebiotic supplements for AD patients (Table 1).

Table 1. Types of dietary fibers associated with the growth of certain gut microbiota.

Fiber Types	Main Features	Natural/Food Sources	Associated Gut Microbiota	References
Cellulose	Bulking	Plant cell wall	*Ruminococcus* spp. *Bacteroides* spp.	(133–135)
Hemicellulose	Bulking	Plant cell wall	*Butyrivibrio* spp. *Clostridium* spp. *Bacteroides* spp.	(136)
Lignin	Bulking	Plant cell wall	*Bifidobacterium* spp.	(137)
Resistant Starch	Fermentable	Seeds and unprocessed whole grains	*Bifidobacterium* spp. *Ruminococcus* spp.	(138)
Fructan				
Fructo-oligosaccharide (FOS)	Fermentable	Jerusalem artichoke, chicory, and the Blue Agave	*Bifidobacterium* spp.	(139)
Inulin	Fermentable	Wheat, bananas, asparagus, Jerusalem artichoke, and chicory	*Bifidobacterium* spp.	(140,141)
Galacto-oligosaccharide (GOS)	Fermentable	Enzymatic conversion of lactose, added in infant formula	*Bifidobacterium* spp. *Lactobacillus* spp.	(142)
β-glucan	Viscous, Fermentable	Bran of cereals such as oats and barley	*Bacteroides* spp. *Prevotella* spp. *Bifidobacterium* spp.	(143,144)
Pectin	Viscous, Fermentable	Pears, apples, berries, and oranges	*Bifidobacterium* spp. *Lactobacillus* spp. *Enterococcus* spp.	(145)
Gums (gum arabic)	Viscous, Fermentable	Substances that are secreted from plant cells in response to injury (gum arabic)	*Bifidobacterium* spp. *Lactobacillus* spp. *Bacteroides* spp.	(146)

Cellulose and hemicellulose are major water-insoluble, non-starch polysaccharides found in plant cell walls. Cellulose degradation is known to be conducted by *Ruminococcus* spp.

and *Bacteroides* spp. producing SCFAs as a byproduct (133–135). Some species of gut microbes, including *Butyrivibrio* spp. *Clostridium* spp. and *Bacteroides* spp. are observed to break down hemicellulose (136). Lignin is also a water-insoluble, non-starch polysaccharide that constitutes plant cell walls together with cellulose and hemicellulose, however its interaction with gut microbes is not well-documented. One study has shown that lignin supports the prolonged survival of bifidobacteria in an in vitro condition (137). Resistant starch, another type of dietary fiber that is water-insoluble is a starch polysaccharide which is not degradable by the α-amylase enzyme of the host. Resistant starch was shown to increase the ratio of Firmicutes to Bacteroidetes (147). At the genus level, *Bifidobacterium* and *Ruminococcus* have been identified to relatively thrive when exposed to resistant starch (138).

Fructan is a polymer of five carbon membered ring fructose molecules, which consists of several different types depending on the chemical bond. Fructo-oligosaccharide (FOS) and inulin are major forms of fructan considered as dietary fibers that are capable of being fermented by multiple members of the gut microbiota community (148). FOS is a short chain oligosaccharide of fructose linked by β (2→1) glycosidic bonds. Inulin is a heterogeneous polysaccharide with β (2→1) linkage and terminal glucose. These fructan molecules have a bifidogenic effect that enhances the relative abundance of *Bifidobacterium* spp. in the host gut (139–141,149). Similarly, galacto-oligosaccharide (GOS) is a short chain polymer of mainly galactose linked with a β (1→4) bond and terminal glucose (150). FOS and GOS are commercially used to produce infant formula to mimic the properties of human milk (151). These oligosaccharides are important nutrients to develop the gut microbiome of infants leading to colonization of beneficial bifidobacteria (152,153). The promotion of these gut

microbiota in infants decreases the niche for pathogenic bacteria and helps to enhance gut barrier function (142,154–156). FOS supplementation in chronically stressed mice was demonstrated to prevent intestinal barrier impairment and neuroinflammation along with improved depression-like behavior and significant changes in the abundance of *Lactobacillus reuteri* (157). FOS from *Morinda officinalis* were also tested in rats with AD-like symptoms and mice with inflammatory bowel disease showing the potential of FOS as a prebiotic that improved gut barrier integrity, alleviated neuronal degradation, downregulated AD markers, and maintained the diversity and stability of the gut microbiome of the host (158).

Beta-glucan is a polysaccharide that contains β-D-glucose linked by glycosidic bonds. A linear, non-branched β-glucan mostly found in the bran of cereals such as oats and barley is water-soluble and consists of β-D-glucose with (1→3), (1→4)-linkage (159). This physicochemical property of β-glucan results in increased viscosity and a thickening effect on feces, and it provides beneficial, saccharolytic gut microbes with fermentable substrate to consume (160–162). Consumption of high molecular weight β-glucan increased the proportion of *Bacteroides* and *Prevotella* (143). Supplementation of either whole grain oats or oat bran elevated the production of SCFAs and produced a bifidogenic effect (144).

Pectin is a water-soluble dietary fiber mainly found in the skin of apples. Pectin is a component of the primary cell wall and middle lamella which contribute to adherence of adjacent plant cells. The structure of pectin is very complex and the pectic polysaccharides are abundant in galacturonic acids. Homogalacturonan is a polymer of galacturonic acid bonded with α-1,4-linkage and the types of pectin may vary according to its side chain sugars (163). These complex pectins are known to be degraded by gut microbiota whose diversity is found to

be preserved by pectin in ulcerative colitis patients (164). Pectins derived from apples were found to be utilized by beneficial colonic bacteria including *Bifidobacterium*, *Lactobacillus*, *Enterococcus,* suggesting a prebiotic capacity of pectin (145).

Gums are commonly found in food thickeners because of their capability of gel formation and emulsion stabilization. Particularly, gum arabic is well determined for its solubility in water, becoming viscous depending on its concentration. Gum arabic is a complex heteropolysaccharide mainly containing 1,3-linked β-D-galactose units with 1,6-linked β-D-galactose side chains attached to rhamnose, glucuronic acid and arabinose residues (165,166). It is accessible to the gut microbes having a potential to increase probiotic bacteria in the human gut. At a dose of 10 g for 4 weeks gum arabic resulted in significantly higher numbers of *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* spp. in a human clinical trial (146).

The structural complexity of dietary fibers and the associated diversity of gut microbes that consume them require further research. It is important to determine the utilization of specific fibers by distinct microbiota and to demonstrate which structural traits and/or components of these fibers affect cognitive function via altering the gut microbiome in future studies.

4. Effectiveness of Prebiotics in Modulating Gut Microbiome Composition and Microbial Metabolite Production

The overall impact of the gut microbiome on the production of microbial metabolites and gut barrier function is summarized in Figure 3.

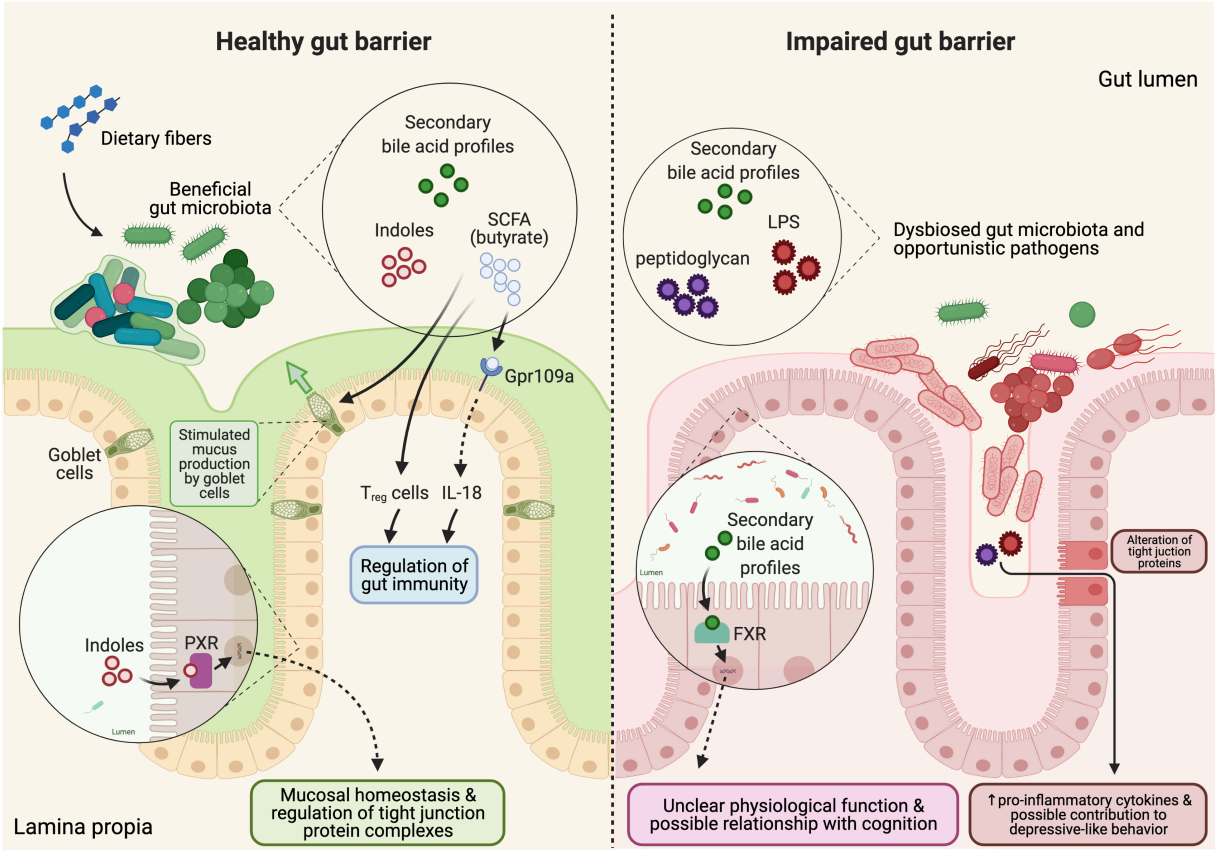


Figure 3. Gut barrier integrity changes and differences in signaling molecules in healthy vs. unhealthy gut.

In healthy gut barrier, dietary fiber from diet is digested by the beneficial gut microbiota which produces secondary metabolites such as SCFA (butyrate), indoles, and secondary bile acid profiles. Butyrate is known to use Gpr109a as a receptor expressed in the enterocyte which produces IL-18, or it may directly affect T regulatory (T_{reg}) cells. IL-18 and T_{reg} cells can both regulate gut immunity. SCFA also stimulates mucus production by goblet cells for healthy mucosal barrier. Indoles are ligands for pregnane X receptor (PXR) acting as transcription factor in sustaining mucosal homeostasis and regulation of tight junction complexes. Secondary bile acid profiles are ligands for farnesoid X receptor (FXR) and can be found in both healthy and unhealthy gut. The physiological roles of secondary bile acid profiles are unclear and may have possible relationship with cognition. In impaired gut barrier, gut microbiota are dysbiosed and byproducts such as peptidoglycan and LPS are released from opportunistic pathogens. The mucosal barriers are attenuated which provides more close contact of pathogens near enterocytes altering tight junction proteins. The peptidoglycan and LPS may pass through compromised tight junction increasing pro-inflammatory cytokines and possibly contributing to depressive-like behavior.

The fermentation of dietary fiber or prebiotics by gut microbiota and the major metabolites from that process have been elucidated in many studies (167–170). Particularly, butyrate is the preferred energy source of apical colonocytes (171). Furthermore, SCFA lower the pH of the gut, suppressing the growth of pathogens (172), mediate gut immune regulation (173), and influence gut motility (174). Thus, SCFAs act as signaling molecules that induce downstream pathways modulating the physiology, immunity, and metabolism of enterocytes. Gpr109a is a type of G protein-coupled receptor specifically activated by butyrate and is expressed in enterocytes, immune cells, and even in microglia (175–177). Butyrate binding to the gpr109a receptor triggers several cellular signaling pathways (Figure 3) including those involving the colonic epithelium, macrophages, and dendritic cells. For example, Gpr109a signaling is known to promote anti-inflammatory properties by inducing IL-18 and IL-10 production, which induces differentiation of naïve T cells to T regulatory cells, thus supporting overall gut immunity by preventing colonic inflammation (178).

Neurotransmitters are another class of signaling molecule that plays an important role in the gut-brain axis. Serotonin, for example, is known to be mostly released from epithelial enterochromaffin cells (179,180). The gut microbiota play a key role in promoting serotonin synthesis by host enterochromaffin cells. SCFA or secondary bile acids produced by gut microbes mediate serotonin production by enterochromaffin cells, which can further affect gut motility via the enteric nerve and brain serotonergic systems (181,182). These findings suggest that certain prebiotic supplements, which stimulate the production of SCFA and secondary bile acids by specific microbes, can improve neurological function and behavior via upregulation of

serotonin (183). Another interesting neurotransmitter that connects gut and brain function is Gamma-aminobutyric acid (GABA). GABA is a crucial inhibitory neurotransmitter in the central nervous system and its alteration in GABAergic mechanisms is related to central nervous system disorders (184). A recent study demonstrated the link between the gut microbiome (*Bacteroides* spp.) and GABA production, a response negatively correlated with depression (185). Fecal microbiota from healthy control and schizophrenia patients were compared and each were transplanted to germ-free mice. Gut microbial dysbiosis shown in schizophrenia was related to changes in the GABA cycle which, in turn, may affect neurobehavioral status such as schizophrenia-relevant behaviors (186). The production of neurotransmitters, particularly serotonin and GABA was distinctly linked with *Bifidobacterium* and *Lactobacillus* genera (187). These findings highlight the potential role of prebiotics that promote the composition of these specific microbes, because their presence has been linked with decreased dysbiosis in the gut and the production of functional neurotransmitters, which may contribute to enhancing enteric health and attenuating AD-related neurobehavioral disorders.

In addition to neurotransmitters, prebiotics may also play an important role in regulating cytokine expression. Soluble fiber (pectin) treatment in mice resulted in faster recovery from endotoxin-induced sickness behaviors along with changes in the concentrations of cytokines, including IL-1RA, IL-4, IL-1β and TNF-α in the brain (188). The pectin-supplemented mice also had increased concentrations of cecal acetate, propionate, and butyrate as a byproduct of pectin fermentation, which was associated with increased gastrointestinal IL-4 (188). These findings suggest that soluble fiber not only affects the gastrointestinal tract and peripheral immune system but also neuroimmune system function. In another study in adult

and aged mice a high fiber diet with inulin led to increased levels of cecal SCFA production including butyrate and acetate (189). A reduction in inflammatory infiltrate was observed in the aged mice on the high fiber diet, and researchers specifically showed that sodium butyrate had anti-inflammatory effects on microglial profile, lowering inflammatory gene expressions (189). These data suggest that butyrate produced from prebiotic fermentation may be a potent modulator of gut immune function and directly linked to microglial function in the brain.

Gut microbiota derived metabolites such as SCFA and indole are critical for sustaining intestinal barrier function (Figure 3). Acetate and butyrate, for example, improve goblet cell differentiation and stimulate mucus production by goblet cells to maintain healthy mucosal barrier (190). Mice fed a low-fiber Western style diet were found to have a defect in mucin production, which was prevented by supplementation with a synbiotic of *Bifidobacterium longum* and inulin (191), suggesting that when SCFA-producing microbes are present in the gut along with a preferred substrate, the net effect is enhanced mucosal barrier function. In addition to a decrease in fiber-fermenting microbes and thus SCFA production, a diet deficient in fiber can also promote the enrichment of mucus-degrading gut microbes such as *Akkermansia muciniphila* (192). *Bifidobacterium bifidum,* which has the ability degrade mucin (193) may protect thinning of the mucus layer by inhibiting *Akkermansia muciniphila,* as was shown in mice with omeprazole-induced small intestine injury (194). Paradoxically, the presence of *Akkermansia muciniphila* has been linked with beneficial health effects (195–198), as well as negative health effects in individuals with certain health conditions (199,200). The roles of specific microbes and their metabolites in the maintenance vs. degradation of the mucosal barrier are context-specific and require further study. Prebiotics may be a useful

strategy to prevent mucus degradation by supporting the growth of SCFA-producing microbes and thus increasing mucin production, as well as sustaining the homeostasis of mucolytic vs. non-mucolytic bacteria in the gut.

Butyrate is known to regulate the expression of tight junction protein complexes (201). Sodium butyrate was shown to increase Claudin-1 expression and induced redistribution of ZO-1 and Occludin in vitro (202). Butyrate treatment accelerated the assembly of tight junctions by reorganizing the tight junction proteins in a Caco-2 cell monolayer model (203). No studies have demonstrated a direct link between butyrate derived from the gut on tight junctions supporting endothelial cells that form the blood–brain barrier. However, these findings of a beneficial effect of butyrate on barrier function in the gut epithelium raises the question of whether a similar benefit may also be found in endothelial cells. A link between butyrate and brain function has been suggested. Bourassa et al. hypothesized that butyrate could be used as an important alternative energy substrate in the Alzheimer's brain where glucose utilization has been found to be reduced (204–206).

Indoles are a class of molecules produced by gut microbes that have the potential to affect gut and brain function. In a germ-free mouse model, oral administration of indole led to up-regulation of tight and adherens junction-associated molecules in the epithelial cells of the colon (207). Indole 3-propionic acid acts as a ligand for pregnane X receptor and increased expression of junctional protein-coding mRNAs while decreasing TNF-α in a mouse model (208). The effect of indole 3-propionic acid was also tested in the Caco-2/HT29 coculture model and showed an increase in tight junction proteins, mucins, and goblet cell secretion products (209). However, the role of indole and its derivatives is controversial in terms of the gut-brain axis

(210,211). Studies have demonstrated potent neuroprotective properties of indoles, which cross the blood–brain barrier and protect the brain from oxidative stress (212) as well as prevent electron leakage from neuronal mitochondria (213,214). However, other studies report excessive production of indole by gut microbes may negatively affect emotional behavior in rats due to the neurodepressive properties of oxidized derivatives of indole, oxindole and isatin (215). Indoxyl sulphate, an oxidized and sulphated form of indole produced from the liver, may reduce the efflux of neurotransmitters through the organic anion transporter 3, causing accumulation of metabolites (216,217). Thus, the effects of indoles on gut barrier and brain function require further study, as the variety of indole metabolites produced by the gut microbes and their co-metabolism by the host generate a complex suite of molecules with differential effects.

Bile acids are a category of metabolite that is modulated by gut microbial metabolism, and which may have effects on the gut-brain axis. Bile acids are produced in hepatocytes and play a critical role in fat digestion and absorption. Most (95%) bile acids are recycled back to the liver via enterohepatic recirculation after reaching the terminal ileum. However, bile acids that are not recycled are excreted in feces or may be metabolized by the colonic microbiota, forming secondary bile acids via a series of microbial enzyme activities including deconjugation and 7α-dehydroxylation (218). Thus, secondary bile acids are gut microbe-derived metabolites that may further regulate bile acid signaling of the host, affecting the activation of the enteroendocrine bile acid receptor, farnesoid X receptor (Figure 3) (219). Several papers have shown a connection between bile acid metabolism and AD. In AD patients, significantly lower serum concentrations of a primary bile acid (cholic acid) and increased secondary bile acid

(deoxycholic acid) were observed compared to cognitively normal older adults (220). Increased deoxycholic acid to cholic acid ratio is known to be strongly associated with cognitive decline (221). The ratio of primary to secondary bile acids was positively correlated with the abundance of *Bifidobacterium* in a human clinical trial (47). Recently, alteration in bile acid profiles was shown to have an association with cognitive decline and AD-related genetic variants (220).

There are likely hundreds if not thousands of microbially produced molecules that likely play important roles in host health. Among these, butyrate, indole, and bile acids, are to date, the most well-studied, and their roles in gut health, brain function, and specific roles in the pathophysiology of AD, are starting to emerge. As we gain knowledge on both short-term and long-term effects of diet on the brain mediated by the gut microbiome, it will be important to establish a dossier of evidence of benefit of specific prebiotics for the pathophysiology of AD. In the following section, we discuss potential prebiotic approaches to supplement AD patients.

5. Current Evidence for Effectiveness of Prebiotics in AD Animal Models and Human Trials

The effectiveness of prebiotics for the treatment of AD will ultimately need to be evaluated on the basis of their ability to either improve or prevent cognitive decline. However, other symptoms of AD related to behavioral and emotional changes are also viable targets of prebiotic intervention studies in AD patients. The current literature showing the potential effects of prebiotics on cognitive function in both animal models and human studies mainly focuses on the effects of fructans, both in the form of oligosaccharides and inulin, β-glucan from yeast or the bran of cereals, plant polysaccharides, and polysaccharides synthesized from sugars. This evidence is summarized below.

5.1. Animal Models

Animal models have been used in several studies to evaluate the effect of prebiotics on AD, particularly mice due to their reliability on intervention and ease of sampling. In this section, animal studies on administration of prebiotics that led to improvement in AD associated brain disorders are summarized. Bimuno-GOS intake in pregnant mice affected the offspring's exploratory behavior and brain gene expression as well as reducing anxiety (222). Additionally, fecal butyrate and propionate levels were increased after Bimuno-GOS supplementation in postnatal mice (222). In another study, behavioral testing was performed on mice from the least stressful (three-chamber test) to the most stressful (forced swim test) for 5 weeks during a 10-week prebiotic administration period including lead-in and lead-out periods (223). The prebiotic treatment with a FOS+GOS combination resulted in a reduction of stress-related (depression and anxiety) behaviors, and reversed chronic stress (elevations in corticosterone and proinflammatory cytokine levels) in the supplemented mice compared to the control mice with no prebiotic treatment (223). In a rat model exhibiting oxidative stress, mitochondrial dysfunction, and cognitive decline in the brain induced by high fat diet-induced obesity these outcomes were improved and cognitive function was restored by 12-week supplementation of either prebiotic (xylo-oligosaccharide), probiotic (*Lactobacillus paracasei* HII01), or combined treatment with similar efficacy (224). The effectiveness of mannan-oligosaccharide was tested in a 5xFamilial AD transgenic mouse model (225). The treatment with mannan-oligosaccharide reduced Aβ accumulation in the brain and suppressed neuroinflammatory responses (225). Mannan-oligosaccharide not only improved cognitive and behavioral disorders, but also gut barrier integrity by reshaping the composition of gut

microbiota, specifically increases in the relative abundances of *Lactobacillus* and decreases in *Helicobacter* (225). Importantly, the observed changes in gut microbiota composition and butyrate production were negatively correlated with oxidative stress in the brain and behavioral deficits (225).

5.2. Human Trials

Studies on the effects of prebiotic supplementation directly on cognitive and behavioral outcomes in Alzheimer's patients are currently lacking. However, a few human intervention studies were conducted to test the effectiveness of certain prebiotics alone or with probiotics on improving symptoms associated with AD such as behavioral, mood, memory, anxiety, and cognitive disorders.

Fructan and GOS-based prebiotics show promising and consistent results in clinical trials in decreasing anxiety and improving cognitive and behavioral outcomes. The prebiotic Bimuno-GOS improved antisocial behaviors in autistic children (226). Trans-GOS stimulated bifidobacteria in the gut of irritable bowel syndrome patients and lowered anxiety (227). Short chain FOS enhanced fecal bifidobacteria and reduced anxiety scores (228). Inulin in healthy participants resulted in better recognition and improved recall (229). In obese patients adhering to calorie restrictions for 3 months supplementation with 16 g/d of inulin had moderate impact on mood and cognition, with responders who experienced an increase in *Coprococcus* and *Bifidobacterium* having stronger benefits than non-responders (230). Importantly, in most of these intervention studies, subjects supplemented with fructan or GOS prebiotics showed increases in bifidobacteria in general along with improvement in their symptoms. Many studies have already reported the connection between the increase in bifidobacteria and beneficial

health outcomes (Table 1). Indeed, the growth of bifidobacteria is selectively stimulated by fructans (231). The increase in *Bifidobacterium longum* 1714 strain in healthy mice showed stress resistance and pro-cognitive effects (232,233). The same *Bifidobacterium* strain from this preclinical study displayed association with reduction in stress and improvement in memory in healthy volunteers (234). The results from these studies suggest a strong connection between prebiotics, the gut microbiome, particularly bifidobacteria, and brain function.

Other studies provide supporting evidence that prebiotics modulate brain function in a manner that would be consistent with desired improvements in symptoms of AD but were not necessarily linked with or did not examine gut microbiome composition. Beta-glucans from yeasts, plants or cereals have been shown to have beneficial health effects on the profile of mood state in healthy individuals (235,236). Plant polysaccharides, which mainly consist of non-starch polysaccharides found in foods were shown to have effect on healthy adults, improving their recognition and memory performance (237,238). Polydextrose, which is a synthesized prebiotic, was supplemented in healthy females and showed moderate improvement in cognition as well as significant change in abundance of *Ruminiclostridium 5* compared to the placebo group (239). Other studies have found 30–60 mL of lactulose for 3 months improved cognitive function and health-related quality of life in patients with minimal hepatic encephalopathy (240).

6. Concluding Remarks

Although human clinical studies examining the effects of specific prebiotics on gut microbiome-mediated cognitive health outcomes in AD patients are lacking, there is mounting

evidence that prebiotics have the potential to be a viable approach for ameliorating symptoms associated with AD. Promoting the growth and activity of beneficial, SCFA-producing microbes such as bifidobacteria is emerging as a clear therapeutic target for improving gut barrier function, decreasing inflammation, and improving cognitive and behavioral outcomes. A variety of prebiotic types, particularly fructans, have been found to be effective in modulating gut microbiome composition and microbial metabolite production, and modifying health outcomes relevant for individuals with AD. More research is needed to determine which prebiotics, at what dosages, and in which context (e.g., on what dietary background, in combination with specific probiotics, at what frequency, etc.) are the most effective for not only decreasing AD-associated symptoms such as anxiety and depression, but also potentially improving cognition or preventing the loss of cognitive function in individuals at risk for AD. Further mechanistic research to determine how changes in the gut microbiome related to prebiotic supplementation alter neuroinflammatory signaling are also needed so that targeted, effective, potentially personalized therapies can be developed to treat and prevent the progression of neurodegenerative processes in AD.

Chapter 3. Metagenomic Analyses Reveal Specific Alterations in Substrate Utilization and Choline Metabolism Which Are Reflected in Plasma Metabolomic Changes in Human Participants Supplemented with a Bifidogenic Prebiotic Supplement

1. Introduction

Many Americans consume diets that are considered to be low in fiber, for reasons that range in nature from financial constraints to personal taste preferences. Dietary fibers are polymers of monosaccharides which are resistant to human digestive enzymes, and which have been shown to have beneficial effects on metabolism including improvements in glucose, insulin levels, and reduction of blood cholesterol levels (86,241,242). Dietary fiber intake is also associated with increases in the overall diversity of the gut microbiome (243) and in the abundance of beneficial microbial taxa, i.e., *Bifidobacterium* (88). The presence of bifidobacteria in the gut microbiome is associated with multiple health benefits including short chain fatty acid (SCFA) production (243,244) and improved gut barrier functionality (192), which in turn are linked with reduced inflammation (245), reduced concentrations of circulating lipopolysaccharide (246,247), and improved gut immune function (248,249). Despite the fact that the daily consumption of dietary fiber is associated with beneficial health effects, the average daily intakes of dietary fiber in the U.S. are only approximately 16 g, which is less than half of the amount recommended by the USDA dietary guidelines (250). There are many practical obstacles that individuals face when considering lifestyle changes that are necessary to increase the intake of dietary fiber. Many Americans do not have access to fresh produce, do not have the time or knowledge to prepare meals containing fiber-rich foods, or simply cannot afford to do so (251–254). Thus, practical solutions are needed to increase fiber intake in

individuals who are consuming low-fiber diets, and who are not able to implement the necessary lifestyle changes that increase fiber intake through whole foods.

Prebiotics are non-digestible carbohydrates present in foods such as whole grains, vegetables, fruits, and legumes which have been shown to beneficially modulate the gut microbiome (255). Prebiotics including inulin, fructooligosaccharides, galactooligosaccharides, and resistant starch are known to increase the abundance of beneficial gut microbiota particularly bifidobacteria in the gut (138,139,141,142). The increased abundance of saccharolytic microbes is in turn associated with enhanced production of microbially derived secondary metabolites that improve overall gut and metabolic health (256). The beneficial effects of SCFA on gut barrier integrity, gut immune function, and overall metabolism have been extensively documented (175,176,178,256–260). Other microbially produced metabolites with potential beneficial and deleterious effects on human health have been identified utilizing targeted and untargeted metabolomic approaches. For example, the metabolite indolepropionate (IPA) has been associated with beneficial health effects (261–263), whereas the metabolite trimethylamine-N-oxide (TMAO) has been linked with and increased risk for cardiovascular disease (264,265).

In this study, we tested the effectiveness of a prebiotic supplement formulation on the gut microbiome and human metabolome in 20 participants using a randomized order, double-blinded, placebo controlled cross-over study design. We hypothesized that 4 weeks of daily supplementation with a targeted bifidogenic prebiotic will increase the abundance of bifidobacteria and the abundance of gut microbial genes associated with bacterial utilization of the prebiotic substrate, alter fecal SCFA concentrations and gut microbial genes related to the

production of SCFA, modify plasma metabolites, specifically, increase IPA and decrease TMAO, and alter cardiometabolic profiles.

2. Methods

2.1. Participants

Twenty-four healthy men and women, aged 18–45 y, BMI of 23.0–32.0 kg/m^2, with a habitual diet low in fiber (< 15 g/day) were recruited at the Ragle Human Nutrition Center, University of California (UC), Davis. Recruitment began in April of 2019 and the study completion date was December of 2019. Exclusion criteria included for screening were: smoking, having anemia and difficulty with blood draws, use of probiotic or prebiotic formulations within 6 weeks of the study start date, use of antibiotics within 6 months prior to study commencement, use of medication such as statins, blood pressure medications, and other prescription medications, pregnancy, use of hormonal birth control in the last 6 months or plans to change or start use of hormonal birth control during the study period, allergies to any placebo or prebiotic ingredients, presence of illness (flu/cold in the last two weeks), presence of documented chronic diseases, presence of intestinal diseases (irritable bowel syndrome, celiac disease, or any inflammatory bowel disease including Crohn's Disease and/or Ulcerative colitis), presence of any immunosuppression symptoms at any point during the study or study enrollment, consumption of > 1 alcoholic drink/day or frequent binge drinking (> 3 alcoholic drinks in one episode) of > 1 day/month, plans to change or recent significant changes in lifestyle (e.g. diet, exercise routine, or major travel), recent weight fluctuations (greater than 10% in the last six months), regular use of over-the-counter pain medications (> 1/week), use of

prescription lipid medications or other supplements known to alter lipoprotein metabolism such as isoflavones, recent medical procedure such as surgery within the last 6 months, and any changes in the above during the course of the study.

The sample size was determined based on a previous study which included 25 healthy participants supplemented with inulin-type fructan-diet to observe a significant increase in bifidobacteria (266). The study was approved by the Institutional Review Board of UC Davis. Written consent was provided by all participants prior to entry into the protocol. One participant was withdrawn due to difficulty with blood draws, 3 participants were withdrawn due to noncompliance with inclusion criteria, and 20 participants (10 male and 10 female) completed the study (Figure 4). This clinical trial was registered at clinicaltrials.gov as NCT03785860.

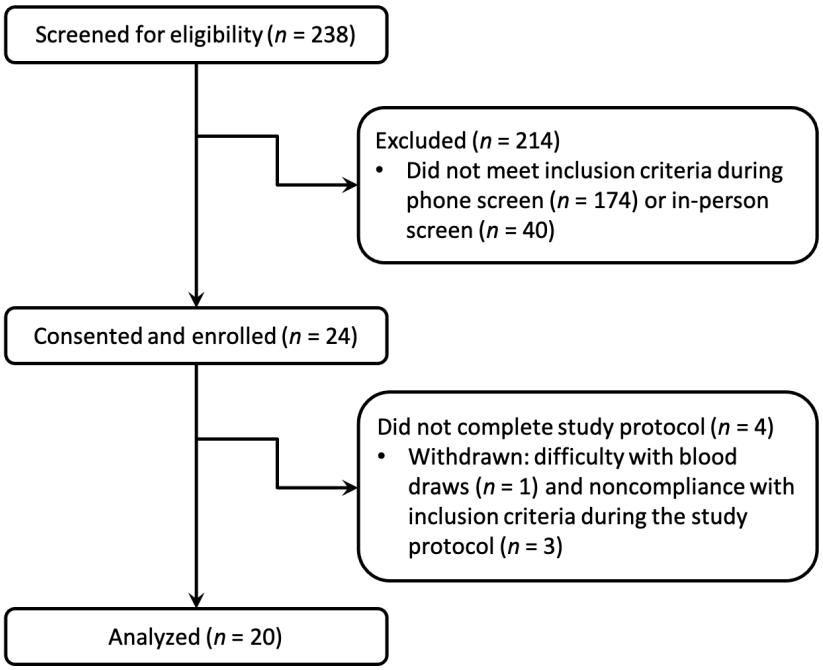


Figure 4. Study CONSORT diagram.

2.2. Study design

The study was a double-blinded, randomized order, placebo-controlled, cross-over design. All participants consumed a prebiotic and placebo supplement for a period of 4 weeks each, with a 4-week washout between intervention arms in random order. Ten participants were randomly assigned to the prebiotic blend as their first intervention arm and 10 were assigned to the placebo blend as their first intervention arm. After completion of the first intervention arm and the 4-week washout, the participants switched to the other intervention arm.

Participants recorded their diet for 3 days using 24-hour diet records at each 2-week segment throughout the duration of the study. The diet was patternized each week, meaning that participants were asked to consume the same meals and foods for the 3 days prior to each test day without significantly changing their usual diet, in an attempt to stabilize the background diet during the days leading up to fecal sample collection, since it has been observed previously that dietary fluctuations over the preceding 3-4 days can significantly influence the gut microbiome (47,267). Diet records were analyzed by nutrition software (Food Processor SQL version 11.7; ESHA).

Questionnaires and anthropometric measurement data were collected at each visit. The SF-12® Health questionnaire was collected to track the general health status of participants during the study intervention. Bowel type and bowel movement frequency, which was rated by the participants throughout the study, was evaluated using the Bristol stool scale (268) and modified bowel movement questionnaire (269). Height was measured with a wall-mounted stadiometer (Ayrton Corp.) and body weight was measured with a calibrated electronic scale

(Scale-Tronix; Welch Allyn). Blood pressure was measured with an automated sphygmomanometer (OxiMax; Welch Allyn) in a seated position. All measurements were performed in triplicate and the average was used for anthropometric measurement data.

The prebiotic packets contained 12 g/serving per day as a powder containing resistant starch, fructooligosaccharide, sugarcane fiber, and inulin while the placebo packets contained 12 g/serving per day of a powder that matched the prebiotic supplement in taste and appearance. The powder packet was mixed with water for consumption. Participants were asked to record daily consumption of the powder packet and to bring back the empty packet for verification of compliance with study protocols.

2.3. Blood sample collection and analysis of cardiometabolic profiles

Whole blood samples were collected after a 12-hour overnight fast at the beginning and end of each intervention arm. Blood samples were collected in EDTA, SST, and PST tubes (Becton Dickinson) via venipuncture by a certified phlebotomist. The samples collected in EDTA tubes were immediately centrifuged (Sorvall-Legend RT) at 1500 x g, 4°C for 10 min. Plasma samples were aliquoted immediately after centrifugation and stored at −80°C until further analyses. Blood samples collected in PST tube were immediately centrifuged at 1300 x g, 4°C for 10 min. The samples in SST tube were allowed to sit for 30 min for the blood to clot and then were centrifuged at 1300 x g, 4°C for 10 min. Samples collected in PST and SST tubes were sent to UC Davis Medical Center Pathology Lab for analyses of glucose, lipid profiles, and insulin. Glucose was analyzed using the glucose oxidase method measuring absorbance at 652 nm after the peroxide catalyzed reaction (270). Insulin was analyzed with the Abbott Architect i1000

chemiluminescent microparticle immunoassay (271). A lipid panel (total cholesterol (TC), triacylglycerols (TG), HDL-cholesterol and calculated LDL-cholesterol) analysis was performed by using a clinical analyzer (DXC 800; Beckman Coulter). TC, TG, and HDL-cholesterol were directly measured, and LDL-cholesterol was calculated using the Friedewald equation (272).

2.4. Metabolomics

Untargeted metabolomic analysis was performed at Metabolon (Morrisville, NC, USA) as previously described (273). All samples were maintained at −80°C until processing. Briefly, individual samples were subjected to methanol extraction then split into aliquots for analysis by ultrahigh performance liquid chromatography/mass spectrometry (UHPLC/MS). The global biochemical profiling analysis comprised of four unique arms consisting of reverse phase chromatography positive ionization methods optimized for hydrophilic compounds (LC/MS Pos Polar) and hydrophobic compounds (LC/MS Pos Lipid), reverse phase chromatography with negative ionization conditions (LC/MS Neg), as well as a HILIC chromatography method coupled to negative (LC/MS Polar) (274). All the methods were alternated between full scan MS and data dependent MS^n scans. The scan range varied slightly between methods but generally covered 70–1000 m/z. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards (275).

2.5. Stool sample collection

Fecal samples were self-collected by study participants. Prior to each stool sample collection day, participants were given stool collection kits consisting of a preservative-filled fecal collection tube (OMNIgeneGUT, OMR-200, DNA Genotek, Ottawa, ON, Canada), fecal collection sheet (Easy Sampler, EU version, GP Medical Devices, Holstebro, Denmark), and sanitizing kit. Trained study personnel educated participants on the stool collection protocol in person prior to the start of the study, and written instructions were also provided in the collection kits to ensure proper collection protocols were followed and to minimize sample contamination and deterioration. Participants were instructed to immediately transfer collected stool samples to a portable cooler packed with frozen icepacks and bring in the collected samples as soon as possible within 24 hours of sample collection including a transfer time of 4 hours at most. Upon arrival to the laboratory, the samples were aliquoted into Eppendorf tubes and immediately stored at −80°C until analysis.

2.6. DNA extraction, sequencing, pre-processing, and assembly

Stool samples were processed at Diversigen (Houston, TX, USA) and metagenomic sequencing was performed following DNA extraction. Briefly, DNA extraction from raw stool samples was performed with PowerSoil Pro (Qiagen) automated for high throughput on the QiaCube HT (Qiagen), with using Powerbead Pro (Qiagen) plates with 0.5 mm and 0.1 mm ceramic beads. Samples were quantified with Qiant-iT Picogreen dsDNA Assay (Invitrogen). Libraries were prepared with a procedure adapted from the Nextera Library Prep kit (Illumina).

Libraries were sequenced on an Illumina NovaSeq using single-end 1x100 reads (Illumina). DNA sequences were filtered for low quality (Q-Score < 30) and length (< 50), and adapter sequences were trimmed using cutadapt (v.1.15). The sequences for each sample were assembled into contigs using SPAdes (v3.11.0). Contigs greater than 1,000 bases in length were used in a QUAST (QUAST v4.5) analysis.

2.7. Gene Annotation and Taxonomy Inference

Prokka (v 1.12) was used to annotate genes for each strain using the contigs > 1,000 bases as described above. Annotation files were parsed and combined to make gene content comparison tables. For taxonomy inference, trimmed and quality filtered sequences were aligned to every reference sequence in CoreBiome's Venti database using fully gapped alignment with BURST (v1.00). Ties were broken by minimizing the overall number of unique gene, Operational Taxonomic Units (OTUs), hits. For taxonomy assignment, each input sequence was assigned the lowest common ancestor that was consistent across at least 80% of all reference sequences tied for best hits. The three most abundant taxa were reported for each strain. Annotated genes for each strain were aligned against the MegaRes (Version 1.0.1) and Virulence Factor Database (VFDB_setB_nt, 10/2018) using BURST at 95% identity. Strain coverage tables were generated in term of the fraction of unique regions covered and the fraction of the overall genome covered for each strain in each sample. The strain coverage tables were used to filter OTUs from the raw OTU table. Samples with fewer than 10,000 sequences were discarded. OTUs accounting for less than one millionth of all strain-level markers and those with less than 0.01% of their unique genome regions covered (and < 0.1% of

the whole genome) at the species level were discarded. The number of counts for each OTU was normalized to the OTU's genome length for downstream analysis.

2.8. Functional annotation and profiling

For Functional annotation and profiling, Kyoto Encyclopedia of Genes and Genomes Orthology groups (KEGG KOs) were observed directly using alignment at 97% identity against a gene database derived from the strain database used above (Diversigen Venti database). The KEGG Orthology group table was filtered to the same subset of samples as the filtered taxonomic tables and used for downstream analysis.

2.9. SCFA analysis

Stool samples were processed at Microbiome Insights (University of British Columbia Vancouver, BC, Canada). Briefly, the SCFA extraction procedure is similar to that of Zhao et al (276). Material was resuspended in MilliQ-grade H_2O, and homogenized using MP Bio FastPrep, for 1 minute at 4.0 m/s. 5M HCl was added to acidify fecal suspensions to a final pH of 2.0. Acidified fecal suspensions were incubated and centrifuged at 10,000 RPM to separate the supernatant. Fecal supernatants were spiked with 2-Ethylbutyric acid for a final concentration of 1 mM. Extracted SCFA supernatants were stored in 2-mL GC vials, with glass inserts. SCFA were detected using gas chromatography (Thermo Trace 1310), coupled to a flame ionization detector (Thermo). A column used for SCFA detection was Thermo TG-WAXMS A GC Column (30 m, 0.32 mm, 0.25 µm) with a flow rate of 6.0 mL/min. The flame ionization detector was set to 240°C with controlled amounts of hydrogen: 35.0 mL/min, air: 350.0 mL/min, and makeup

gas (Nitrogen): 40.0 mL/min. SCFA standards were acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and hexanoic acid from Sigma Aldrich.

2.10. Statistical Analysis

A statistical analysis plan was generated prior to data analysis. Statistical analyses were performed with the package edgeR (277) in R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Linear mixed models were used for each outcome with fixed effect of treatment, treatment order, and their interaction and random effect of individual participants. Each participant was given an individual slot, and the treatment and timepoint interaction were tested for significance ($P < 0.05$). The primary outcome of the study was the change in the relative abundance of *Bifidobacterium* associated with the prebiotic supplement compared to the placebo. A negative binomial regression with the linear mixed model was performed to evaluate the effect of treatment on *Bifidobacterium* levels against placebo. The quasi-likelihood F-test was applied to calculate and determine the differences in abundance at the genus level between the treatment and placebo arms. The secondary outcomes of the study were changes in other microbe abundances as well as gene counts, cardiometabolic profiles, SCFA concentrations, anthropometric measurements, and plasma metabolomic profiles. Specifically, we hypothesized that the gene counts of *sacA*, *xfp*, *xpk*, *poxB*, *ackA*, and *buk* genes would alter on the prebiotic arm. Also, we hypothesized that the concentrations of indolepropionate would increase, whereas TMAO would decrease on the prebiotic arm. A linear mixed model with the same design formula mentioned above was applied to each of the secondary outcomes, with Shapiro-Wilk normality test performed prior to downstream analysis.

Multiple test adjustment was performed whenever more than 10 variables were tested using the Benjamini–Hochberg method. Kendall's correlation was performed to study novel relationships between changes in OTU counts of genus *Bifidobacterium* and changes in gene counts of bacterial genes as well as metabolomic profiles. Gene set enrichment analysis (GSEA) was performed to test the effect of prebiotic treatment on metabolic pathways which include genes enriched by the treatment against the placebo. Partial least-squares discriminant analysis (PLS-DA) was performed to investigate the effect of the prebiotic treatment on overall metabolomic profiles.

3. Results

3.1. Anthropometrics, cardiometabolic profiles, and diet records

Participant baseline characteristics and their cardiometabolic profiles pre and post the prebiotic and placebo arms are summarized in Table 2. No significant changes were found in anthropometric measurements. Cardiometabolic profiles showed no significant differences between prebiotic and placebo groups. Also, there were no significant changes in nutrient intake at any time point (Table 3). No significant changes were observed for bowel type and bowel movement frequency during the study period (data not shown).

Table 2. Participant anthropometric and cardiometabolic characteristics pre- and post-treatment on placebo and prebiotic*

Variable	Placebo		Prebiotic		*P* value
	pre	post	pre	post	
Age, y	27.1 ± 6.1	27.1 ± 6.1	27.1 ± 6.1	27.1 ± 6.1	NA
Weight, kg	74.8 ± 10.4	74.8 ± 10.1	75.5 ± 10.2	74.9 ± 10.7	0.29
Height, cm	169.3 ± 9.0	169.3 ± 9.3	169.3 ± 9.1	169.4 ± 9.2	0.84
BMI, kg/m^2	26.1 ± 2.9	26.1 ± 2.9	26.3 ± 2.9	26.1 ± 2.8	0.17
SBP, mmHg	113.5 ± 7.3	112.8 ± 7.7	112.8 ± 6.5	113.9 ± 6.7	0.36
DBP, mmHg	74.2 ± 4.7	73.7 ± 5.0	74.1 ± 5.4	74.4 ± 5.5	0.44
Fasting glucose, mg/dL	83.6 ± 6.3	85.8 ± 7.7	86.2 ± 7.0	85.3 ± 7.1	0.29
Fasting insulin, µIU/mL	6.3 ± 3.3	6.9 ± 3.8	6.2 ± 3.9	6.1 ± 3.2	0.42
TG, mg/dL	73.1 ± 40.8	64.0 ± 31.6	69.4 ± 37.1	69.0 ± 29.9	0.38
TC, mg/dL	176.2 ± 23.6	175.1 ± 21.3	176.1 ± 25.9	172.3 ± 25.1	0.61
LDL cholesterol, mg/dL	109.2 ± 21.9	107.7 ± 19.9	109.4 ± 21.7	106.2 ± 22.1	0.71
HDL cholesterol, mg/dL	52.4 ± 12.1	54.5 ± 15.0	52.8 ± 12.2	52.4 ± 11.9	0.12
TC:HDL cholesterol	3.5 ± 0.8	3.4 ± 0.8	3.4 ± 0.6	3.4 ± 0.7	0.55
Non-HDL cholesterol, mg/dL	123.8 ± 22.8	120.6 ± 21.6	123.3 ± 22.9	120.0 ± 23.1	1.00

*Data are shown as means ± SDs. Changes on pre- and post-treatment with placebo or prebiotic were compared with a linear mixed model (*n* = 20).

SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triacylglycerol.

Table 3. The composition of background diet of participants pre- and post-treatment with placebo and prebiotic*

Variable	Placebo		Prebiotic		*P* value
	pre	post	pre	post	
Total kcal	2182.2 ± 634.7	1866.7 ± 592.9	1871.2 ± 655.9	2008.5 ± 811.4	0.09
Carbohydrate, g	245.1 ± 96.1	215.2 ± 121.7	212.5 ± 85.3	220.9 ± 129.4	0.36
Protein, g	101.9 ± 56.0	84.8 ± 30.8	85.9 ± 44.5	89.8 ± 39.6	0.13
Fat, g	89.5 ± 38.9	75.1 ± 28.3	76.0 ± 38.5	84.3 ± 42.5	0.16
Total dietary fiber, g**	18.0 ± 7.9	14.4 ± 9.5	13.5 ± 6.4	13.2 ± 7.7	0.21

*Data are shown as means ± SDs. Changes on pre- and post-treatment with placebo or prebiotic were compared with a linear mixed model (*n* = 20).

**Prebiotic supplement was not included in the background dietary intake of total dietary fiber.

3.2. Gut microbial composition

The overall gut microbial abundance and diversity were calculated using the Shannon diversity index to measure alpha diversity of microbiome species in samples and the Bray-Curtis dissimilarity index to evaluate beta diversity of species difference between the placebo and prebiotic intervention arms. There were no significant changes in microbial diversity after the prebiotic intervention compared to the placebo (Supplementary Figure 1A-C). These results show that interindividual variability had more impact on the diversity than the treatment effect.

Further analyses on relative abundance of all gut microbiota in fecal samples were performed from phylum to species levels. The relative abundance of the phylum Actinobacteria significantly increased after the prebiotic ($P = 0.03$) compared to the placebo arm (Figure 5A). Under the phylum Actinobacteria, the family Bifidobacteriaceae significantly increased after the prebiotic ($P = 0.002$) compared to the placebo arm (Figure 5B). *Bifidobacterium* counts were significantly increased after the prebiotic treatment ($P = 0.005$) compared to the placebo at the genus level (Figure 5C), confirming the primary hypothesis. In addition, several species under the genus *Bifidobacterium* increased on the prebiotic arm compared to the placebo arm (Figure 5D-H), including *Bifidobacterium bifidum* ($P = 0.01$), *Bifidobacterium adolescentis* ($P = 0.02$), *Bifidobacterium breve* ($P = 0.03$), *Bifidobacterium catenulatum* ($P = 0.03$), and *Bifidobacterium longum* ($P = 0.04$).

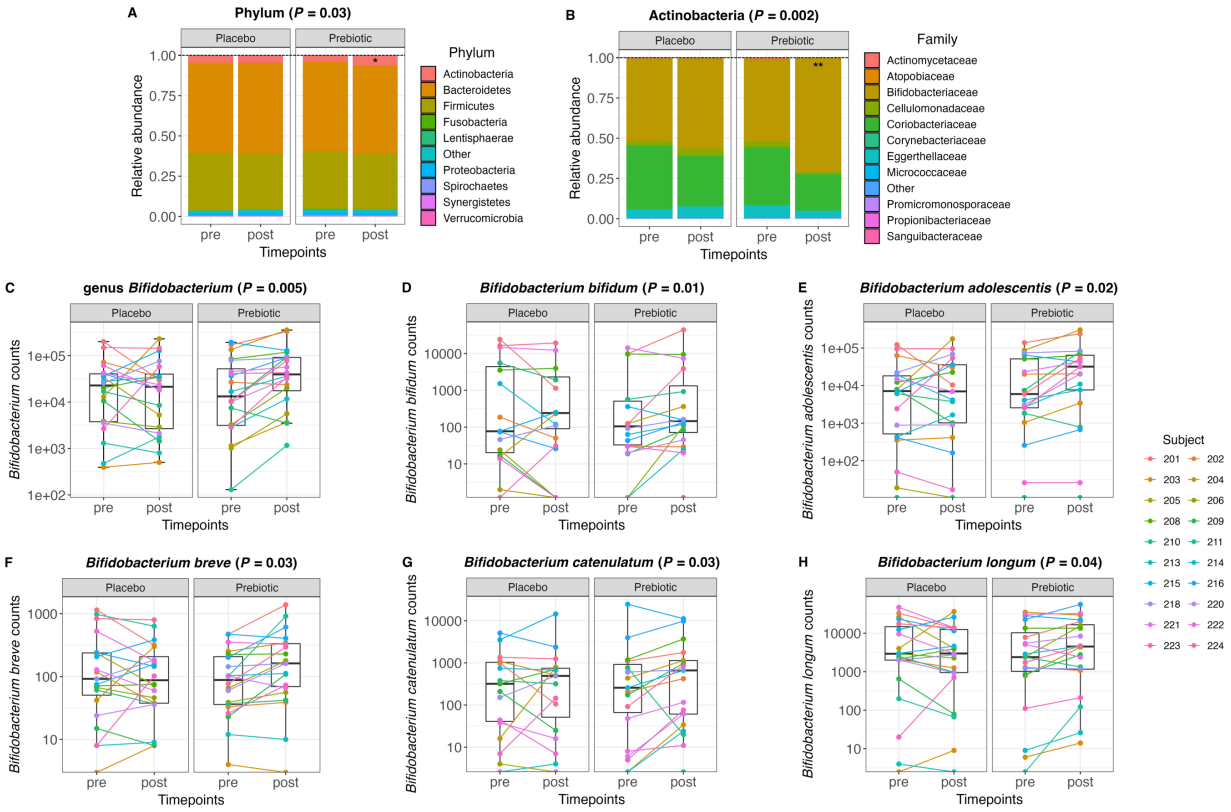


Figure 5. The changes in relative abundance and *Bifidobacterium* counts of the gut microbiome community pre- and post-treatment with placebo or prebiotic.
A and B, Bar plots of the relative abundance of the gut microbiome at phylum level and at family level under phylum Actinobacteria pre- and post-treatment with placebo or prebiotic. C, Box plots of the genus *Bifidobacterium* counts pre- and post-treatment with placebo or prebiotic. D-H, Box plots of the species count under *Bifidobacterium* pre- and post-treatment with placebo or prebiotic (*$P < 0.05$, **$P < 0.01$).

The overall composition of the gut microbiota that increased or decreased after the prebiotic supplement compared to the placebo is shown in the circular cladograms (Supplementary Figure 2A, B). The only phylum that significantly changed after the prebiotic supplement was Actinobacteria. At the genus level (Supplementary Figure 2A), 11 genera significantly ($P \leq 0.05$, unadjusted) changed with the prebiotic treatment compared to the placebo. Among these genera, genus *Bifidobacterium*, *Anaerostipes*, and *Hungatella* increased

(in red), respectively, while the other genera decreased (in blue). At the species level (Supplementary Figure 2B), 22 species significantly ($P \leq 0.05$, unadjusted) changed with the prebiotic treatment compared to the placebo. However, none of these changes remained statistically significant after correction for multiple testing.

3.3. Gut microbial metagenome

Several genes increased (in blue) and decreased (in red) after the prebiotic supplementation compared to the placebo (Figure 6A). Overall, 163 out of 2,718 genes significantly ($P \leq 0.05$) increased or decreased after the prebiotic arm. Among the genes that changed, 49 genes decreased, and 114 genes increased with the prebiotic treatment.

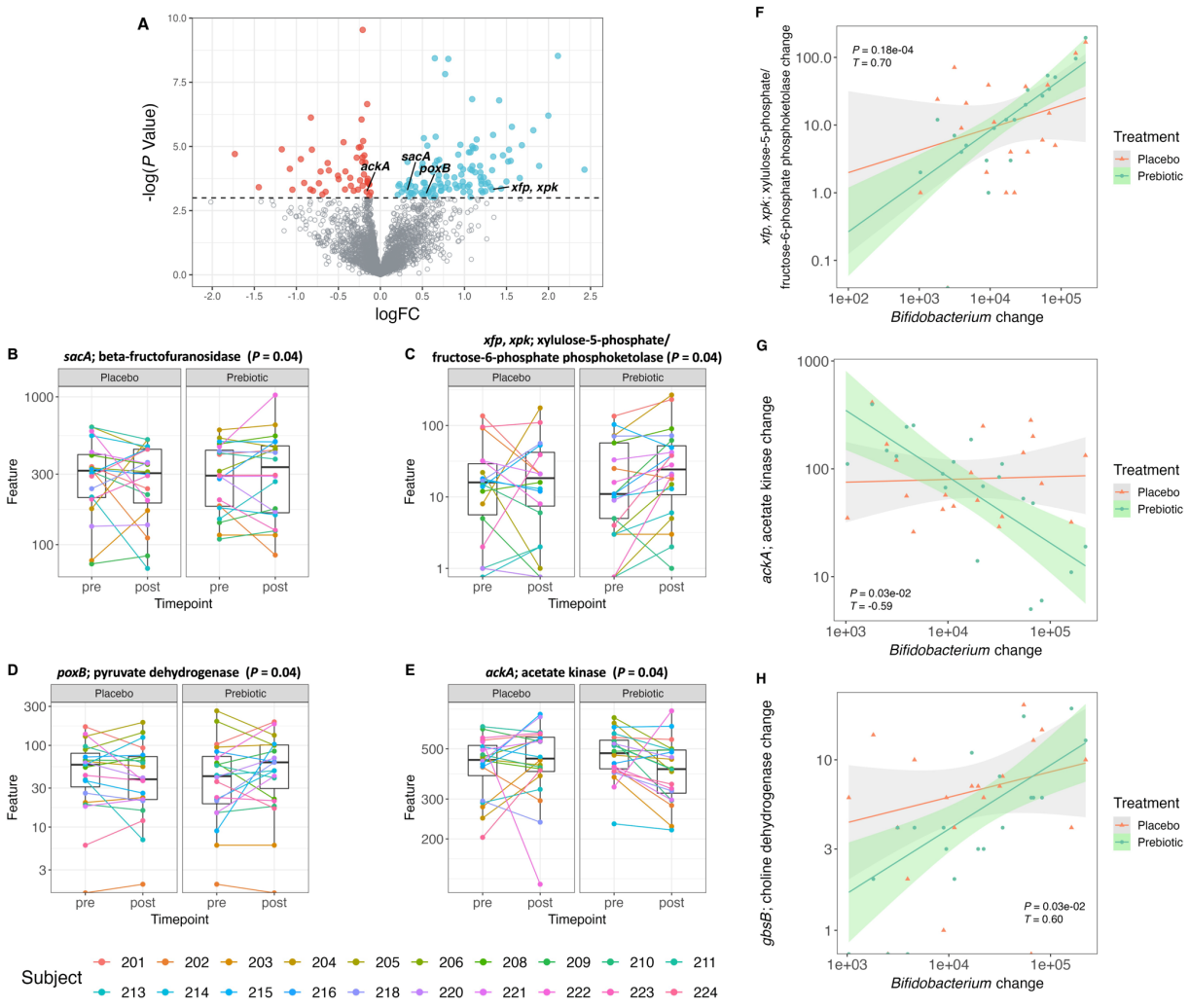


Figure 6. The metagenomic analysis on the changes in gene counts of genes and their correlation with changes in *Bifidobacterium* OTU counts.

A, Volcano plot of all genes in the human gut microbiome. Genes that had logFC > 0 and −log(*P* Value) > 0.05 were colored in blue and genes that had logFC < 0 and −log(*P* Value) > 0.05 were colored in red. All the other genes were colored in gray. B-E, Box plot of gene counts of genes pre- and post-treatment with placebo or prebiotic ($P \leq 0.05$, unadjusted). F-H, Correlation plot on changes (post−pre) in OTU counts of *Bifidobacterium* against changes (post−pre) in gene counts of genes for both placebo and prebiotic in 20 subjects (prebiotic: $P \leq 0.05$, adjusted, Kendall *T*).

The pathways involved in the bacterial genes were analyzed by gene set enrichment analysis (GSEA) (Supplementary Figure 3A). The dot plot shows the number of genes associated

with the specified metabolism pathway by count size and color range of p-values for each pathway. The plot displays the Gene Ratio (number of significant genes related to KEGG pathway / total number of significant genes) by the size of dots. There were 10 pathways that were significantly ($P \leq 0.05$, unadjusted) enriched by the prebiotic supplementation (Supplementary Figure 3A).

Starch and sucrose metabolism and pentose phosphate pathway were two enriched pathways associated with the genes that we specifically hypothesized to increase in their counts after the prebiotic supplement compared to the placebo. The gene involved in starch and sucrose metabolism that showed significant increase after the prebiotic treatment was *sacA* coding for beta-fructofuranosidase (Figure 6B). This enzyme produces fructose and glucose from sucrose (278). The gene associated with fructose utilization was found to be significantly increased after the prebiotic supplement which was mostly fructose-based sugars (Figure 6C). The genes *xfp*, *xpk* encoding for xylulose-5-phosphate/fructose-6-phosphate phosphoketolase were shown to be associated with pentose phosphate pathway converting D-xylulose-5-phosphate to D-glyceraldehyde-3-phosphate as well as D-fructose 6-phosphate to D-erythrose-4-phosphate (279). The phosphoketolase reaction on xylulose-5-phosphate and fructose-6-phosphate is one of the major reactions found in the bifidobacteria shunt (280,281). Bifidobacteria utilize the hexose sugars to generate ATP producing SCFAs as byproducts (282).

As certain bifidobacterial species are known for their ability to produce SCFA such as acetate and butyrate, we specifically hypothesized genes, *poxB*, *ackA* and *buk*, associated with the production of these SCFAs to increase in gene counts after the prebiotic supplement compared to the placebo. Pyruvate to acetate conversion is mediated by the gene *poxB*

encoding for pyruvate dehydrogenase (283). Acetyl phosphate to acetate conversion is mediated by the gene *ackA* encoding for acetate kinase/phosphotransacetylase (283). Butyryl phosphate to butyrate conversion is mediated by the gene *buk* encoding for butyrate kinase (284). In this study, the gene, *poxB*, related to SCFA production, specifically acetate formation (285), was increased after the prebiotic supplement (Figure 6D). Interestingly, the *ackA* gene counts were shown to decrease after the prebiotic supplement compared to the placebo (Figure 6E). The gene counts of other genes (*pta*, *acs*, and *buk*) in bacterial SCFA production pathways did not change after the prebiotic supplementation (data not shown).

As we observed an increase in the relative abundance of bifidobacteria after the prebiotic treatment (Figure 5), we performed a treatment-stratified correlation analysis between changes (post−pre) in OTU counts of *Bifidobacterium* and changes (post−pre) in gene counts of genes (Supplementary Figure 3B) in order to ascertain which gene count changes were attributable to the increase in bifidobacterial abundance. The changes in bifidobacterial OTU counts were significantly correlated with the changes in gene counts from the prebiotic arm but not with the placebo arm. Among 30 genes, 29 genes were positively correlated, and 1 gene was negatively correlated with change in *Bifidobacterium* (Supplementary Figure 3B). In the correlation analysis of change in *Bifidobacterium* vs. gene count change, the gene *sacA* did not remain significant after multiple testing adjustment (Supplementary Figure 3B). However, the correlations between changes in *Bifidobacterium* and changes in the gene counts of *xfp* and *xpk* did remain statistically significant after multiple testing adjustment (Figure 6F). A negative correlation between changes in *Bifidobacterium* and changes in *ackA* gene counts also remained statistically significant after multiple testing correction (Figure 6G). Additionally, the

gene counts for *gbsB*, a choline dehydrogenase, increased (though it did not reach statistical significance) ($P = 0.07$) after the prebiotic treatment compared to the placebo, and the changes in the gene counts of *gbsB* were positively correlated with changes in *Bifidobacterium* (Figure 6H).

3.4. Gut microbe-derived metabolites

3.4.1. Plasma metabolites

Overall plasma metabolites that significantly ($P \leq 0.05$, unadjusted) increased (in blue) or decreased (in red) after the prebiotic included 45 out of 889 metabolites (Figure 7A). We hypothesized a priori that plasma concentrations of IPA would increase whereas TMAO would decrease as an effect of prebiotic supplementation. The results of differential expression analysis of each metabolite are shown in Figure 7B-C. The prebiotic supplement formulation increased the total amount of IPA ($P = 0.04$) compared to the placebo. However, the supplement had no significant effect on the total amount of TMAO ($P = 0.84$) compared to the placebo.

We observed significant decreases in choline and its acylated derivatives, the acylcholines. The plasma concentrations of stearoylcholine, dihomo-linolenoyl-choline, linoleoylcholine, arachidonoylcholine, palmitoylcholine, oleoylcholine, and choline significantly decreased after the prebiotic intervention compared to the placebo (Figure 7D-J).

We further sought to determine whether any plasma metabolites were corelated with abundances of *Bifidobacterium*. A heatmap was generated to determine metabolites that are either positively or negatively associated with *Bifidobacterium* (Supplementary Figure 4A).

Twenty-six metabolites were positively associated with *Bifidobacterium* and 6 metabolites were negatively associated with *Bifidobacterium* with adjusted p-values less than 0.05 after multiple testing correction. A total of 6 metabolites that significantly changed after the prebiotic treatment were also correlated with *Bifidobacterium* (Supplementary figure 4A). Additionally, partial least-squares discriminant analysis (PLS-DA) was performed to test if the prebiotic treatment had a discernible overall metabolomic signature. The prebiotic, while altering bifidobacterial abundance and increasing the concentration of IPA as well as decreasing the concentrations of several acylcholines, did not have an overall effect on the plasma metabolome, as shown by overlap of the time points in the scores plot (Supplementary Figure 4B).

3.4.2. Fecal metabolites

SCFA concentrations in the stool samples were unchanged after the prebiotic treatment in healthy subjects (Supplementary Table 1).

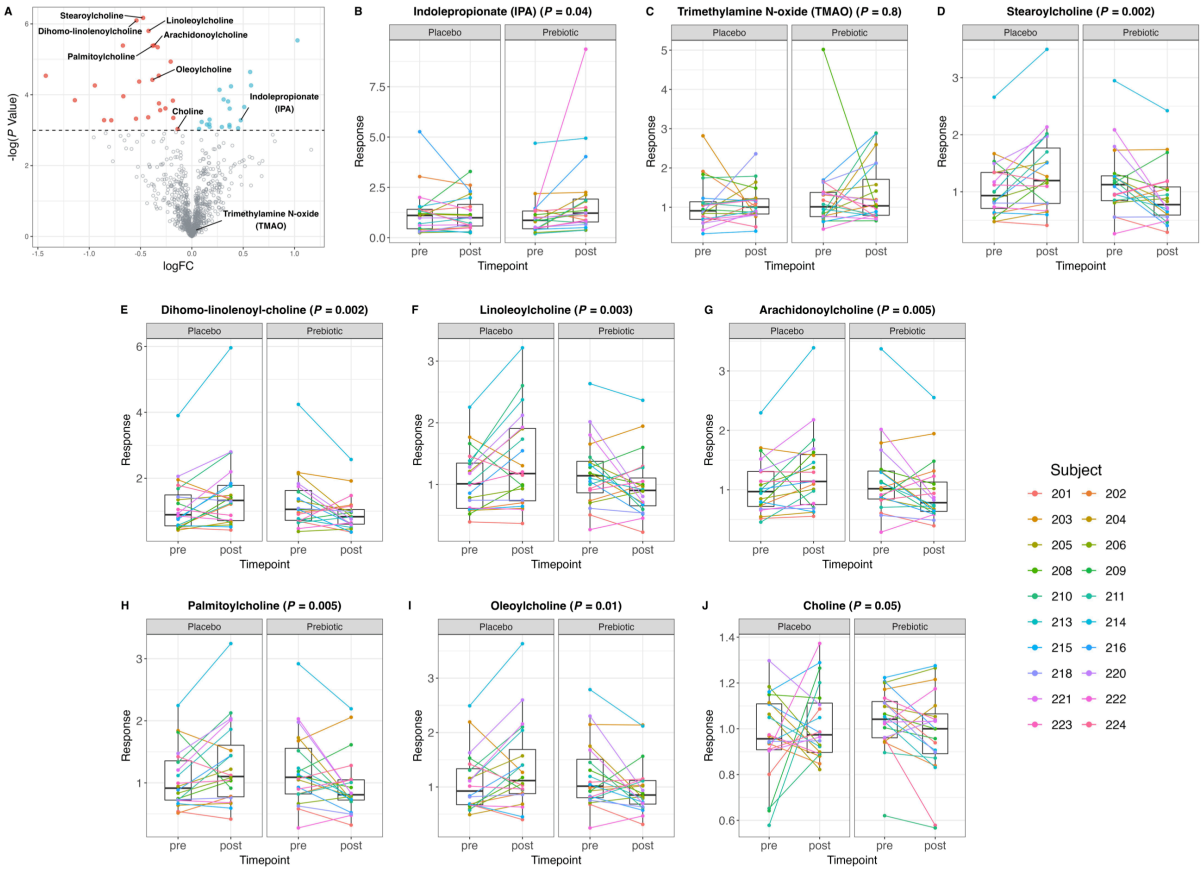


Figure 7. The metabolomic analysis on the changes in concentrations of human plasma metabolites.

A, Volcano plot of all metabolites in human plasma samples. Metabolites that had logFC > 0 and −log(*P* Value) > 0.05 were colored in blue and metabolites that had logFC < 0 and −log(*P* Value) > 0.05 were colored in red. All the other metabolites were colored in gray. B-J, Box plots of IPA, TMAO, choline, and acylcholines concentrations pre- and post-treatment with placebo or prebiotic ($P \leq 0.05$, unadjusted).

4. Discussion

Low intake of dietary fiber in adults is associated with a number of deleterious health effects including an increased risk for metabolic disease and inflammation (286–291). The recommended intake for dietary fiber is 14 g/1,000 kcal per day (292). In this study, the objective was to supplement 12 g/day of a bifidogenic prebiotic formulation to participants consuming low fiber diets (< 15 g/day) to determine whether a convenient, easy to use prebiotic supplement can have a measurable impact on gut microbiome composition by increasing the abundance of beneficial bifidobacteria, and whether such an improvement in the gut microbiome is associated with measurable changes in the plasma metabolome and cardiometabolic profiles in healthy young adults.

As hypothesized, the bifidogenic prebiotic increased the relative abundance of the genus *Bifidobacterium* as well as that of several bifidobacterial species and the phylum Actinobacteria. *Bifidobacterium* species that increased included *B*. *bifidum*, *B. adolescentis*, *B*. *breve*, *B*. *catenulatum*, and *B*. *longum*. These results align with the results of other studies, which demonstrate increases in bifidobacteria after the consumption of fructan-based oligosaccharides (139,141,142,293). Healthy adults who consumed as little as 5 g of inulin for 21 days showed significant increases in *Bifidobacterium* species including *B*. *adolescentis* and *B*. *bifidum* (92). Treatment with 16 g/day of inulin-type fructans for 3 months in obese women resulted in a significant increase in the species *B*. *adolescentis* and *B*. *pseudocatenulatum*, and *B*. *longum* (141).

The prebiotic supplement did not change the overall structure of the microbiome, and a high degree of inter-individual variability in the microbiome was observed, as has been

demonstrated extensively in previous studies (294–299). However, consuming the prebiotic supplement for 4 weeks led to significant changes in the specific bifidobacterial species which were targeted by the prebiotic due to its composition of fructan oligosaccharides, which are known to act as a selective substrate for bifidobacteria. Two other studies supplementing inulin-type fructan prebiotics (15-16 g/d) increased *Bifidobacterium* but did not significantly alter the overall microbial community due to large inter-individual variability (266,300).

In the current study, in addition to assessing the increase in relative abundance of bifidobacteria, we also sought to determine the genetic changes at the level of the metagenome that occur in response to supplementation, in order to elucidate the specific mechanisms by which an increase in bifidobacterial abundance in the gut is associated with functional changes that may confer benefits to the host. We hypothesized that gene counts of genes that code for proteins involved in the transport and metabolism of the oligosaccharides contained within the prebiotic supplement would increase on the prebiotic arm. Specifically, we hypothesized that *sacA,* coding for the enzyme beta-fructofuranosidase, and *xfp*, *xpk*, coding for xylulose-5-phosphate/fructose-6-phosphate phosphoketolase, would both increase in response to the supplement. The beta-fructofuranosidase enzyme converts sucrose into glucose and fructose (278), which is involved in the utilization of glucose and fructose for growth or energy sources by bifidobacteria (301–304). We found that changes in OTU counts of *Bifidobacterium* and changes in gene counts of *sacA* were not significantly correlated. This is likely because many other gut bacteria other than bifidobacteria also express beta-fructofuranosidase to utilize fructose as a substrate (305,306), and thus the increase in the gene counts of this enzyme could not be attributed solely to the increase in bifidobacteria (302,307).

The enzymes xylulose-5-phosphate/fructose-6-phosphate phosphoketolase each converts D-xylulose-5-phosphate to D-glyceraldehyde-3-phosphate and D-fructose 6-phosphate to D-erythrose-4-phosphate (279). The dual enzymatic reaction of these phosphoketolases are related to bifidobacterial utilization of hexose sugars in the 'bifid shunt', producing acetyl phosphate as well as SCFAs as byproducts while generating ATP (281,282). Bifidobacterial species are specifically known for their ability to produce acetate from fructooligosaccharides (308,309). Under anaerobic conditions, such as the human gut, acetyl phosphate is converted to acetate, which is facilitated by acetate kinase encoded by the *ackA* gene (310). Another enzyme, pyruvate dehydrogenase, which produces acetate from pyruvate is encoded by the *poxB* gene (311). In the current study the gene counts of *ackA* decreased and those of *poxB* increased after the prebiotic treatment. This results in an overall increase in the acetate pool, providing substrate for acetyl-CoA (312). The acetyl-CoA may in turn enter the TCA cycle for complete oxidation of sugar molecules in cellular respiration (313). Acetyl-CoA may also be involved in the production of intracellular butyrate (284,314). However, gene counts of the *buk* gene associated with butyrate production and other genes related to the metabolic pathways of SCFA production did not change.

Furthermore, acetate, butyrate, and other SCFA concentrations in the stool samples were unchanged. It is not clear why this increase in acetate production genes was not associated with changes in acetate measured directly in fecal samples. Similar results were found in clinical studies supplementing prebiotic (inulin-type fructans) in healthy individuals (266,300). The utilization of acetate and butyrate by the host as energy source particularly in the colon (315,316) may be one of the contributing factors for the lack of measurable effect on

acetate and butyrate concentrations. Alternatively, the prebiotic supplement at a dosage of 12 g/day may not be sufficient to detect any changes in SCFA concentrations. Lastly, discrepancies in the water content of each fecal sample may have decreased the signal to noise ratio. A recent paper demonstrated that lyophilization of fecal samples reduces detection errors from water content and improves SCFA stability (317). Thus, implementing fecal lyophilization may be able to capture more accurate SCFA concentrations in future studies.

In this study we sought to further elucidate the relationships between gene count changes and the increase in bifidobacterial abundance by performing a treatment-stratified correlation analysis between the change in the OTU counts of genus *Bifidobacterium* and changes in counts of genes. Two genes, *xfp*, *xpk* and *ackA*, were found to have a positive and a negative association, respectively, with *Bifidobacterium*, which were also differentially expressed after the prebiotic supplement compared to the placebo. As discussed previously, *xfp*, *xpk* genes are related to a unique carbohydrate metabolism pathway utilizing the phosphoketolase enzyme in bifidobacteria, known as the bifid shunt (318). The positive correlation between changes in bifidobacterial OTU counts and changes in counts of *xfp*, *xpk* genes may be due to the unique utilization of prebiotic supplement from the intervention. The *ackA*-*pta* pathway and *poxB* pathway are known to have an important role during the exponential growth phase and stationary phase, respectively, in *E. coli* for producing acetate (283). In *E. coli*, a small RNA, SdhX, regulates encoding enzymes of the TCA cycle and represses the expression of *ackA* while *pta* is not significantly affected (319). Many papers show significance of the acetate fermentation genes *ackA*-*pta* in bifidobacteria (319–321).

An interesting gene shown to be positively correlated with bifidobacteria was *gbsB* encoding for choline dehydrogenase. Not much is known about the role of choline dehydrogenase in bifidobacteria, and this may be the first report of a positive correlation between *gbsB* gene and *Bifidobacterium*. A paper showed decreased levels of choline in rats treated with certain strains of *Bifidobacterium* and *Lactobacillus* (322). The most well studied links between choline and gut microbiota are associated with trimethylamine (TMA) production (323–325). The *cut* gene cluster including choline-TMA lyase encoded by the *cutC* gene is expressed by diverse taxa of Firmicutes and Proteobacteria converting choline into TMA (326). Prebiotic supplementation may modulate the gut microbiota that utilize choline and thus affect plasma TMA or TMAO concentrations, as has been shown in obese children (327) and in mice (328). However, the concentration of TMAO did not change after the prebiotic compared to the placebo, confirming a similar lack of effect in other studies (47,265). On the other hand, choline metabolites were found to uniformly decrease after the prebiotic treatment including choline itself and several acylcholines including stearoylcholine, dihomo-linolenoyl-choline, linoleoylcholine, arachidonoylcholine, palmitoylcholine, and oleoylcholine. In colon cancer patients the consumption of rice bran results in a decrease in palmitoylcholine, linoleoylcholine, and oleoylcholine (329). In contrast, infants supplemented with rice bran showed increases in palmitoylcholine, oleoylcholine, linoleoylcholine, and stearoylcholine (330). Long-chain acylcholines have been found to have associations with a number of disease states (331), including elevated concentrations in endometrial cancer patients (332) and patients at high risk of pulmonary embolism (333), and lower concentrations in patients with myalgic encephalomyelitis/chronic fatigue syndrome (334) and chronic thromboembolic pulmonary

hypertension (335). Acylcholines are known to have cholinergic signaling properties, with important implications for signaling through the nicotinic acetylcholine receptor (α7 nAChR), and effects on cytokine synthesis in macrophages and T cells (331,336).

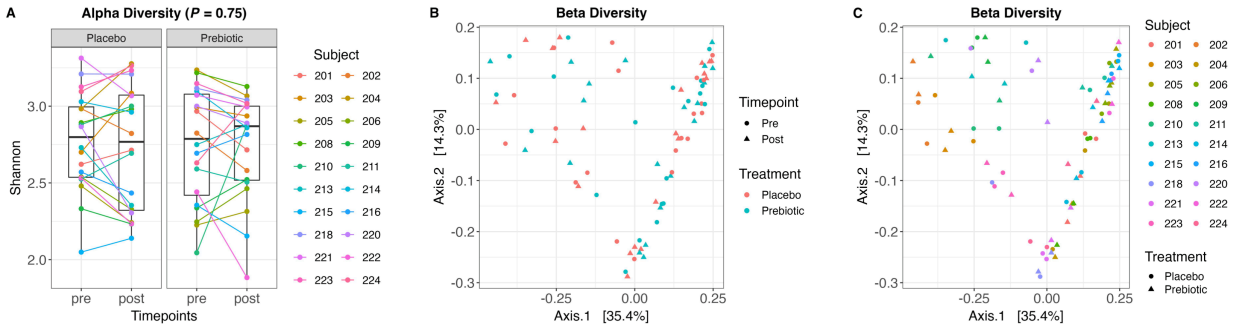
In addition to changes in TMAO we hypothesized that the metabolite IPA would increase after the prebiotic intervention, and indeed, an increase in IPA was observed. Previous studies have shown that the concentration of IPA in the blood was positively correlated with dietary fiber intake (337). A clinical study on healthy individuals who consumed a high-fiber Mediterranean diet also showed an increase in IPA (47). IPA is produced by gut microbes from tryptophan and has been determined to play a crucial role in sustaining mucosal barrier function (207,256,338). Evidence on the beneficial health effects of IPA is growing (212,339,340), highlighting the therapeutic potential of probiotics and prebiotics that increase IPA production.

In this tightly controlled dietary intervention study, great care was taken to maximize the stability of the background diet as much as possible in order to maximize the ability to detect an effect of the prebiotic supplement. Participants were instructed to maintain their habitual diet throughout the course of the 12-week study protocol. We confirmed through 3-day diet record analysis that there were no significant changes in the background diet. This intervention in young, healthy participants who consume a low-fiber diet, but who were nonetheless metabolically healthy, did not affect cardiometabolic profiles. Fasting glucose, insulin, and lipid panels were not significantly altered after the prebiotic supplementation compared to the placebo. Several studies show clinically meaningful decreases in fasting or postprandial blood glucose and insulin concentrations in response to fiber supplementation

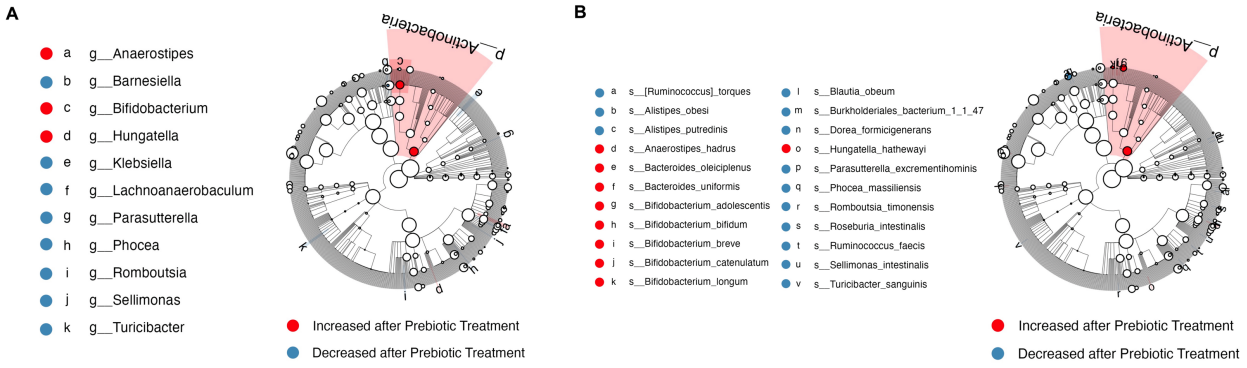
(86,341,342). However, many of these studies were conducted in participants with elevated baseline values of these cardiometabolic parameters, such as individuals with metabolic syndrome (341) or type 2 diabetes (343), or in healthy participants (344,345) but at much higher fiber doses (e.g. 38 g/d vs. 12 g/d in this study) (86). Other studies show results that are in line with our study demonstrating no significant differences in cardiometabolic profiles between groups supplemented with fiber diet vs control diet (48,346).

In conclusion, supplementation with 12 g/day of a bifidogenic prebiotic resulted in measurable increases in beneficial *Bifidobacterium* species, changes in counts of genes associated with the utilization of the prebiotic as well as acetate production, and changes in plasma IPA, choline, and acylcholines in generally healthy individuals who consume a low-fiber diet. These results demonstrate a tangible benefit of even a relatively low amount of prebiotic supplement (12 g/day) in individuals who do not consume recommended amounts of dietary fiber, highlighting that even small, easy to incorporate changes in dietary intake can have beneficial effects on gut microbiome-mediated metabolism.

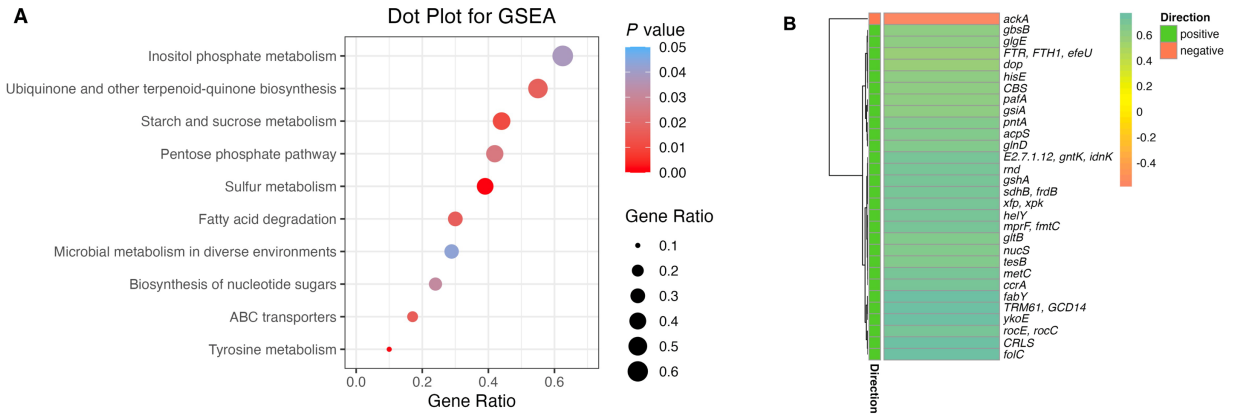
5. Supplementary figures and tables



Supplementary Figure 1. The overall diversity analysis of the gut microbiome community.
A, Shannon alpha diversity of the gut microbiome community pre- and post-treatment with placebo or prebiotic. B and C, Bray-Curtis beta diversity of the gut microbiome community color coded by treatment and subject, respectively.

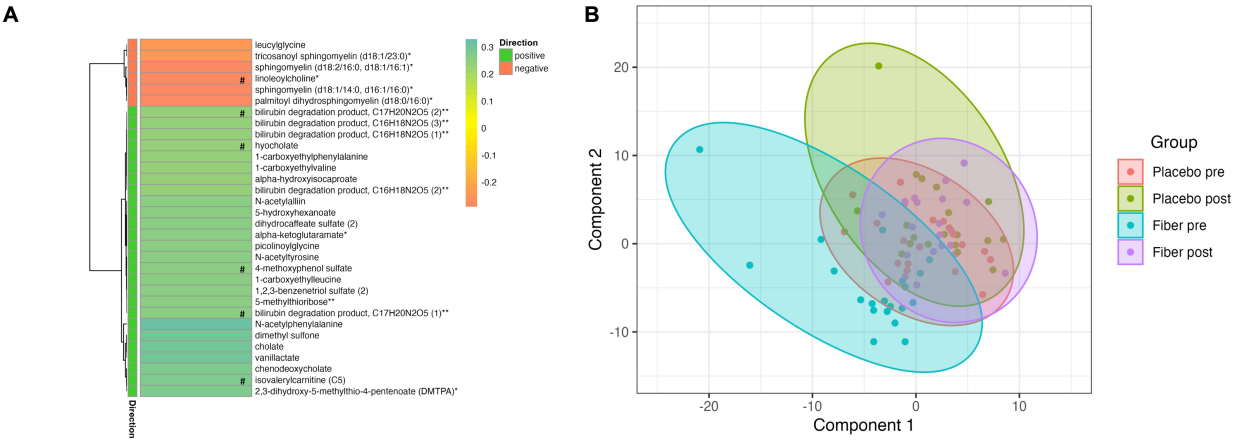


Supplementary Figure 2. Circular cladograms of changes in the overall gut microbiota composition.
A and B, Circular cladograms of the gut microbiota that significantly ($P \leq 0.05$, unadjusted) changed after the prebiotic at genus (A) and species (B) levels.
The red dots indicate microbes that increased, and the blue dots indicate microbes that decreased after the prebiotic treatment.



Supplementary Figure 3. The analysis on gene set enrichment and the correlation heatmap between gene counts and *Bifidobacterium* OTU counts.

A, Dot plot of gene set enrichment analysis. Metabolic pathways enriched by the prebiotic treatment compared to the placebo were calculated and indicated by the color of *P* value (unadjusted) and size of Gene Ratio. B, Heatmap of genes that were positively (green) or negatively (pink) associated with *Bifidobacterium*. The adjusted p-values of 30 genes are less than 0.05 after Benjamini–Hochberg adjustment.



Supplementary Figure 4. The correlation heatmap between plasma metabolite concentrations and *Bifidobacterium* OTU counts. PLS-DA score plot of the plasma metabolome.

A, Heatmap of metabolomic profiles that were positively (green) or negatively (pink) associated with *Bifidobacterium*. The adjusted p-values of 32 metabolites are less than 0.05 after Benjamini–Hochberg adjustment (* suspected metabolites from mass analysis, # metabolites

that were found in both volcano plot and heatmap). B, PLS-DA score plot generated from each treatment and timepoint groups.

Supplementary Table 1. Fecal SCFA concentrations*

SCFA	logFC	Average expression	t	p-value
Acetic Acid	0.011	2.29	0.068	0.946
Propionic Acid	0.505	0.337	1.24	0.219
Isobutyric Acid	-0.180	-1.39	-0.634	0.529
Butyric Acid	0.065	0.869	0.334	0.740
Isovaleric Acid	0.177	-0.510	0.836	0.406
Valeric Acid	0.109	-0.527	0.575	0.568
Hexanoic Acid	0.087	-1.48	0.391	0.697

*Data are presented as log fold change (logFC), average expression across all samples, logFC divided by its standard error (t), and p-values for each SCFA with a differential expression analysis.

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