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Effect of Prebiotic, Probiotic, and Enzyme Supplementation on Gut Fermentation, Markers of Inflammation and Immune Response in Individuals with GI Symptoms

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by

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ABSTRACT

Effect of Prebiotic, Probiotic, and Enzyme Supplementation on Gut Fermentation, Markers of Inflammation and Immune Response in Individuals with GI Symptoms

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Current practices support the use of probiotic and prebiotic supplementation to improve chronic gastrointestinal distress (GID). The aim of this study was to determine the tolerance and benefits of GlutenShield (GS), a prebiotic, probiotic, and enzyme supplement, on adults with GID. Subjects (n=20) took either GS or the placebo for 30 days and completed a pre-treatment FFQ as well as a pre- and post-treatment GID questionnaire, blood draw, and stool sample. Participants consumed more total and saturated fat, and less fiber and whole grains compared to the recommended intake. A significant reduction in IgG2 was observed in the GS group ($p=0.008$) as well as a significant reduction in self-reported bloating ($p=0.038$) with no change observed to cytokines or SCFAs ($p>0.05$). GS was well tolerated and perceived to be beneficial; however, further research is needed to identify the specific population of GID patients who could most benefit from GS supplementation.

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CHAPTER 1

INTRODUCTION

Probiotics and prebiotics are common nutrition supplements consumed by individuals to enhance immune function, attenuate gastrointestinal upset, improve mood and provide a host of other benefits. Currently, probiotic and prebiotic supplements are recommended by physicians, dietitians, and other healthcare providers to mitigate symptoms/side effects associated with certain disease states and medications and are also supplemented to improve quality of life. The purpose of this research is to identify if there is a relationship between GlutenShield, a combination prebiotic, probiotic, and enzyme supplement, and the gut environment of individuals with gastrointestinal upset. The secondary purpose is to understand the effects that probiotics, prebiotics, and enzymes in relation to the claims currently being supported. In an article published by the Academy of Nutrition and Dietetics on eatright.org, Registered Dietitian Taylor Wolfram claims that probiotics "are linked to promoting the growth of helpful bacteria in the gut," "may boost immunity and overall health, especially GI health," and "bottom line: At a minimum, prebiotics and probiotics are keys for good gut health, which affects many other areas of the body."[1] Through this research we provide complete picture of the diet, lifestyle, fermentation, and inflammatory profile of a group of individuals with gastrointestinal upset. From there we investigate the impact that targeted probiotic, prebiotic, and enzyme supplementation has on gastrointestinal symptoms, mood states, fermentation, and markers of immunity and inflammation. Research Objectives are detailed below:

1. To determine if there is a correlation between typical dietary intake and initial fecal short chain fatty acid concentration, markers of inflammation, and predominant gut microbial population.

2. To determine if pre-/probiotic/enzyme supplementation affects short chain fatty acid production in the gut as evidenced by short chain fatty acid concentration in fecal matter to improve digestion, absorption, and bowel function.
3. To determine whether pre-/probiotic/enzyme supplementation will improve serum markers of inflammation, including GM-CSF, IFN-gamma, IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-alpha and IgA and IgG.
4. To determine whether pre-/probiotic/enzyme supplementation will create an improvement in psychosocial measures of self (Profile of Mood States 2nd Edition survey).
5. To determine whether pre-/probiotic/enzyme supplementation alters the predominant gut microbial population in feces.
6. To determine whether pre-/probiotic/enzyme supplementation alters the concentration of microbes in feces.

CHAPTER 2

REVIEW OF LITERATURE

Gastrointestinal Upset

Epidemiology

Symptoms of gastrointestinal (GI) upset include abdominal pain, heartburn, acid regurgitation, bloating, nausea and vomiting, abdominal distension, eructation, increased gas, decreased passage of stools, increased passage of stools, loose stools, hard stools, urgent need for defecation, and feeling of incomplete evacuation.[2] Chronic GI symptoms unexplained by structural or biochemical abnormalities are common in the primary care setting; however, epidemiological data concerning general GI symptoms is generally unavailable for the United States/ North America.[3] In the general global population, the occurrence of chronic GI symptoms is estimated to be 5.3-20.4%.[3] Further, according to Peery et al, abdominal pain resulted in 8,863,568 outpatient visits in 2009, while constipation resulted in 3,980,438 visits and diarrhea resulted in 2,402,350 visits in the United States.[4] In an effort to characterize the prevalence and defining characteristics of GI upset, epidemiological data on constipation and diarrhea is discussed below.

According to a systematic review conducted by Higgins et al, the prevalence of constipation ranges from 1.9% to 27.2% in North America with an average percentage of 14.8. Studies that relied on self-reported constipation documented a prevalence of 27.2 per 100 individuals; whereas, studies that evaluated constipation using the Rome Criteria estimated a lower prevalence of 14.9-16.7 per 100 individuals.[5] Despite the differences in criteria to evaluate and define constipation, studies consistently reported a higher ratio of constipation in females compared to males at 2.2:1.[5] Higgins et al's systematic review indicated that incidences of

constipation gradually increased after age 50 and dramatically increased after age 70.[5] Very few studies evaluate the effects of constipation on perceived quality of life and psychosocial measures. In a survey evaluating health-related quality of life, researchers discovered a significant difference in health related quality of life in individuals with constipation compared to those without constipation.[6] Additionally, women reported a greater impaired quality of life compared to men.[6]

Based on the 2009-2010 National Health and Nutrition Examination Survey (NHANES) bowel health questionnaire, the prevalence of chronic diarrhea in adults in the United States, excluding inflammatory bowel disease and celiac disease, is 6.6%.[7] Similar to constipation, the incidence of chronic diarrhea is 1.4 times greater in females compared to males ($p=0.004$).[7] Also, individuals with a BMI ≥30 and individuals over the age of 70 had a higher prevalence of diarrhea compared to individuals with a BMI ≤29 and individuals between the ages of 20-29 ($p\leq0.005$).[7] Buono et al, using the 2012 National Health and Wellness Survey, found that individuals with chronic diarrhea (irritable bowel syndrome- diarrhea) have a significantly lower quality of life score ($p<0.001$), have higher rates of absenteeism from work ($p<0.001$), have lower work productivity ($p<0.001$), and have higher rates of physical activity impairment ($p<0.001$).[8]

Diet

Research on dietary intake and distribution of calories among individuals with GI symptoms is limited. One such study compared the dietary intake of 99 individuals with functional gastrointestinal disorders (FGIDs) to 119 individuals without symptoms (controls).[9] Using the Bowel Disease Questionnaire to evaluate GI symptoms and the Harvard Food Frequency Questionnaire to evaluate usual dietary intake over a given week, Saito et al found

that consumption of wheat-containing foods, lactose-containing foods, caffeinated drinks, fructose-sweetened beverages, and alcoholic beverages was similar between both groups ($p>0.05$).[9] Further, mean caloric, carbohydrate, protein, fat, and vitamin intake was similar between groups ($p>0.05$).[9] As a percentage of total calories, individuals with FGIDs consumed a higher percentage of calories from fat, saturated fat, and monounsaturated fat compared to controls ($p<0.05$); however, there was no difference in the percentage of calories from polyunsaturated fats ($p>0.05$).[9] When assessing consumption of bioactive substances, there were no differences in the amount of serotonin-containing or tryptophan-containing foods ($p>0.05$).[9] Consumption of epinephrine-containing foods (chocolate, nuts, bananas, oranges, and raisins) was slightly higher in FGID individuals compared to controls (57% vs 45%); however, the difference was not statistically significant ($p=0.10$).[9] Although limited research is available, the results of this study suggest that the diet of individuals with GI symptoms is similar to the diet of individuals without GI symptoms. Interestingly, individuals with FGIDs had similar consumption of foods that are commonly thought to cause GI upset (i.e. lactose, wheat, and fructose).[9]

In contrast to the above study, Torres et al evaluated the diets of 1,870 individuals formally diagnosed with irritable bowel syndrome (IBS) compared to 34,578 healthy individuals (controls) using three 24-hour recalls that were administered randomly over a 6-month period.[10] IBS is a gastrointestinal disorder characterized by abdominal pain and altered bowel habits (diarrhea, constipation, or mixed).[10] Individuals with IBS had a lower consumption of milk ($p<0.0001$), yogurt ($p=0.001$), and fruits ($p<0.001$) compared to controls.[10] Individuals with IBS had a higher total caloric intake compared to controls ($p<0.001$) and IBS subjects were more likely to consume the recommended dietary fiber intake (≥25g/day) compared to control subjects

($p=0.07$).[10] It is generally assumed that there is a positive correlation between certain dietary habits and gastrointestinal upset, however, as the above research studies suggest, there is a high level of heterogeneity in the specific dietary factors that contribute to GI upset.[10] Other factors, such as the human gut microbiome, can influence the GI tract and the presence of GI symptoms.

The Human Gut Microbiome

Schippa et al. compares the human gut microbiome to the intricacy of a fingerprint. Similar to a fingerprint, the colonic microbiome is complex and varies from person to person.[11] The colonic microbiota can contain over 100 different bacterial phyla; further, the composition of the microbiota changes based on one's diet, weight status, environment, geographical location, and health status (i.e. inflammatory and disease status). Understanding these factors allows researchers to better understand the components of a symbiotic, or healthy, gut microbiome as well as the components of a dysbiotic, or imbalanced, microbiome.

Core Microbiome

Research shows that the microbiome can be generalized to an extent. For example, Backhed et al found that the human gut microbiome is dominated by two phyla- *Bacteroidetes* and *Firmicutes.*[12] Zupancic et al supported the predominance of *Bacteroidetes* and *Firmicutes* in the colonic microbiome and also found a significant correlation between the *Bacteroidetes: Firmicutes* ratio and age and sex-adjusted BMI ($p=0.04$).[13] That being said, Zupancic et al observed significant differences in the concentration of both dominant and rare genera in ≥95% of subjects.[13] By sequencing 16S rDNA, Schippa et al supported Zupancic's research. Schippa et al found that the majority of bacterial species detected in the gut belong to the *Bacteroidetes* and *Firmicutes* phyla.[11] Mariot et al observed an average ratio of 10.9 of *Firmicutes* to *Bacteroidetes* in the fecal microbiota of adults.[14] The ratio of *Firmicutes* to *Bacteroidetes* is thought to be an

indicator of gut microbial health.[15] Typically, *Firmicutes* represent 60-80% of the total bacteria within the gut while *Bacteroidetes* represent 15-30% of the total bacteria within the gut.[15]

According to Harmsen et al and Mariat et al, anaerobic fecal bacterial can also be divided into two groups by genus: dominant and subdominant.[14,16] Dominant anaerobic fecal bacteria, which are $>10^9$ coliform forming units (CFUs)/gram, include *Bacteroides, Eubacterium, Bifidobacterium, Peptostreptococcus, Ruminococcus, Clostridium* and *Propionibacterium*.[14,16] Subdominant anaerobic fecal bacteria, which are $<10^9$ CFUs/gram, include *Streptococcus, Enterococcus, Lactobacillus, Fusobacterium, Desulfovibrio* and *Methanobrevibacter* as well as certain bacteria from Enterobacteriaceae family, particularly *Escherichia coli*.[14,16]

Despite the diversity of bacterial species, Schippa et al and Turnbaugh et al argue for a common core gut microbiome, or set of functions that are shared by the majority of gut microbial genes.[11,17] Functions of these core genes include the synthesis of short-chain fatty acids (SCFAs), amino acids and vitamins, as well as the degradation of polysaccharides.[11,17]

Microbial Profile of GI Upset

Physiological etiologies that may lead to GI symptoms include abnormal GI motility, visceral hypersensitivity (heightened pain within viscera), immune dysregulation, inflammation, mucosal barrier dysfunction, alterations in the gut microbiome, dietary intake and variety, maldigestion or malabsorption of nutrients, as well as the amplification or disruption of the gut-brain axis.[18] For the purpose of this review, GI symptoms are discussed in relation to the colonic microbiome and in relation to dietary intake. Alterations of the gut microbiome may not only contribute to GI upset, but GI symptoms may also be a reflection of an altered microbiome.

In a cross-sectional study, Zhu et al found that children with constipation had a significantly different microbial profile compared to children without constipation. Children with

constipation had a significantly higher proportion of several different Firmicutes.[19] Mancabelli et al similarly found that adults with functional constipation (FC), defined as having three or less bowel movements per week, have a different fecal microbiota composition compared to healthy subjects (HS).[20] At a phyla level, FC and HS participants both had an predominance of Bacteroidetes and Firmicutes; however, the abundance differed between groups. At a genus level, FC participants exhibited increased average relative abundance of *Bacteroides* (34.25%) , *Faecalibacterium* (6.85%), *Alistipes* (6.48%), and *Lachnospira (*4.44%) bacteria.[20] HS subjects also had increased relative abundance of *Bacteroides* (45.23%) and *Alistipes* (5.34%). The researchers did not find a specific microbial biomarker associated with functional constipation.[20] That being said, HS participants had a higher concentration of *Bacteroides* ($p=0.0004$), *Roseburia* ($p=0.006$), and *Coprococcus* ($p<0.01$), while FC subjects had higher concentrations of Faecalibacterium ($p=0.0001$) and other genera of the Ruminococcacae family.[20]

Mancabelli et al then selected 10 participants, 5 FC and 5 HS, and performed Ilumina shotgun sequencing.[20] The data collected from shotgun sequencing the samples was used to compare microbial pathways between FC and HS subjects using the MetaCyc database. The researchers found a significant difference in 629 metabolic pathways. HS individuals had a higher number of genes associated with carbohydrate and fatty acid metabolism ($p<0.05$) compared to FC subjects. These pathways influence the production of SCFAs and may provide a defense for the etiology of functional constipation based on one's gut microbiome. In contrast, FC subjects had a higher abundance of methanogenic pathway genes, meaning FC subjects have a predicted higher capacity to produce hydrogen compared to HS subjects ($p=0.05$).[20]

Individuals with diarrhea also display an altered gut microbial distribution. Samb-Ba et al found that adults with diarrhea had a significantly higher number of bacterial species per stool sample compared to adults without diarrhea; however, the individuals with diarrhea had lower proportions of Bacteroides ssp. (including *Bacteroidetes vulgatis*) in comparison.[21] This is supported by Chang et al in a study that evaluated the fecal microbiota of adults with *Clostridium difficile* associated diarrhea through analysis of 16S rRNA gene sequences.[22] Chang et al found that the majority of gene sequences from the control subjects were from the *Bacteroidetes* and *Firmicutes* phyla; however, individuals with C. difficile, an opportunistic pathogen, had an altered distribution of the 16S phylotypes, indicating a deviation from what is recognized as the "normal" microbiome.[22]

Nutrients and the Gut Microbiome

The composition of the human gut microbiota is strongly influenced by the composition of one's diet. Roughly eight hours after consuming a meal, undigested chyme, primarily indigestible polysaccharides, resistant proteins, water and electrolytes as well as endogenous mucins and enzymes, enter the large intestine for further digestion and absorption.[23] Within the colon, water and electrolytes are rapidly absorbed, microbial fermentation occurs, and feces are formed and stored. A diverse microbial community known as the gut microbiome, works to salvage energy and absorbable nutrients, feed the intestinal epithelial cells, and protect the host against invasion by pathogenic organisms.[23] The composition of the gut environment directly affects colonic transit time, pH, osmolarity, and gas production.[24]

Carbohydrates are the major substrates that influence the colonic microbiome. While the majority of carbohydrate digestion and absorption occurs in the small intestine, certain carbohydrates bypass the small intestine and enter the large intestine undigested. These

carbohydrates include plant cell wall polysaccharides (cellulose, arabinoxylan, xyloglucan, b-glucan, mannan, pectins and lignin), resistant starch, inulin, fructo-oligosaccharides, simple sugars and sugar alcohols.[24] Some plant cell-wall polysaccharides, such as cellulose and lignin, are only partially digested in the large intestine, while hemicelluloses and pectins undergo more complete breakdown by colonic bacteria.[23] As an example, Dongowaski et al found that cultures of *Bacteroides thetaiotaomicron* isolated from human fecal flora first degraded pectin to oligogalacturonic acids and then completely fermented the oligogalacturonic acids to SCFAs and gases within 24 hours *in vitro*.[25]

Fructans (i.e. inulin and fructo-oligosaccharides (FOSs)), found naturally in certain fruits (bananas), vegetables (garlic, onions, artichokes), and grains (wheat, barley), have been shown to stimulate the growth of beneficial bacteria in the colon.[26] As such, inulin and FOSs, are commonly referred to as prebiotics. Research shows that different prebiotics have varying capabilities to stimulate the growth of bacteria within the gut. Scott et al demonstrated that short-chain FOS were able to stimulate the growth of several Firmicutes, Actinomycetes, and Bacteroidetes, including *Roseburia*, *Bifidobacterium*, and *Bacteroides* species; however, many bacteria were unable to utilize long-chain FOS (inulin) for growth.[26]

Unabsorbed sugars and sugar alcohols can also be used as substrates for fermentation in the large intestine. In a randomized-crossover study conducted by Vogt et al human subjects ingested 25g of L-rhamnose, lactulose, or d-glucose per day for 28 days.[27] Results from the study showed that L-rhamnose significantly increased serum propionate when compared to lactulose ($p<0.05$), while lactulose supplementation raised the serum acetate: propionate ratio ($p<0.005$).[27] Both results indicate fermentation of 6-carbon sugars; however, the specific microbes responsible for fermentation were not assessed.[27] Different phyla, species, and strains of bacteria

ferment different carbohydrate sources, and, as a result, produce different end-products. Identifying typical carbohydrate sources through use of food recalls can allow researchers to identify the amount and types end-products produced to potentially exert beneficial effects to the host.

Short Chain Fatty Acids

Production and Absorption

Short-chain fatty acids (SCFAs) are the primary end-products of anaerobic bacterial fermentation in the colon. The three major SCFAs – acetic acid (C2), propionic acid (C3), and butyric acid (C4) (structures shown in Table 1) – are produced via carbohydrate fermentation and represent 90-95% of the SCFAs produced in the large intestine.[28] Acetate, propionate, and butyrate are typically produced in a 3:1:1 ratio.[28] Select branched chain fatty acids are also produced via proteolytic breakdown of valine, leucine, and isoleucine.

There is no single metabolic pathway for the production of SCFAs, rather several pathways are used depending on the colonic microbiota composition, the substrates available for fermentation, and other environmental conditions (i.e. colonic pH and level of CO_2 production). These pathways are important to document in order to understand ways to modulate and increase SCFA production by enteric bacteria. This section discusses the metabolic pathways utilized in relation to the end-product produced- acetate, propionate, or butyrate.

Table 1: Properties of the Three Major SCFAs[29,30]

Fatty Acid	Structure	Molecular Weight (g/mol)	Boiling Point (°C)	Melting Point (°C)	Density
Acetic Acid	$CH_3 – COOH$	60.05	118	16.6	1.08
Propionic Acid	$CH_3 – CH_2 – COOH$	74.1	141	-20.7	0.99
Butyric Acid	$CH_3 – (CH_2)_2 – COOH$	88.1	163.5	-5.7	0.96

Acetate is the most abundantly produced SCFA and constitutes more than 50% of the SCFA composition in feces.[30] Acetic acid's structure is a carboxylic acid bonded to a single methyl group (Table 1) (Acetate: $CH_3 – COO^-$). Approximately one-third of acetate is produced by acetogenic bacteria.[28] Acetogenic bacteria generate acetate from hydrogen and carbon dioxide or from formate via the Wood-Ljungdahl pathway.[29] Through the Wood-Ljungdahl pathway, one molecule of glucose will produce 3 molecules of acetate.[30] That being said, the majority of acetate is produced by colonic anaerobes through fermentation of carbohydrates from acetyl-CoA.[31] Anaerobes that produce acetate also utilize reducing equivalents to produce other products such as succinate, propionate, butyrate, formate, d-lactate, l-lactate, and ethanol.[28]

Propionic acid's structure is a carboxylic acid bonded to a methylene group bonded to a methyl group (Table 1) (Propionate: $CH_3 – CH_2 – COO^-$). Colonic bacteria utilize three pathways to produce propionate: the succinate pathway, the acrylate pathway, and the propanediol pathway (Figure 2).[28,31,32] The succinate pathway is the most widely utilized pathway by *Firmicutes*, *Negativicutes* and *Bacteroidetes*, to produce propionate.[31] In this pathway, oxaloacetate from pyruvate goes through the TCA cycle to produce succinate as a substrate for the formation of propionate. The decarboxylation of methylmalonyl-CoA to form propionyl-CoA is specific to the succinate pathway.[32] The second pathway used to produce propionate is the acrylate pathway. In

this pathway, lactoyl-CoA dehydratase and enzymatic reactions convert lactate to propionate.[31] This pathway is utilized by very few Firmicutes (*Veillonellaceae* and *Lachnospiraceae* families) and is not thought to produce a significant amount of propionate in the colon.[31,32] Distinct Firmicutes and Proteobacteria produce propionate via the propanediol pathway.[28] The propanediol pathway is characterized by the conversion of deoxy-sugars (i.e. fucose and rhamnose) to propionate.[28,31,32] Salonen et al conducted a study to evaluate the effects of dietary interventions on SCFA production and bacterial communities.[33] The researchers saw that an increase in fecal Bacteroidetes concentration positively correlated to fecal propionate concentrations.[33] This research suggests that Bacteroidetes (using the succinate pathway) are the primary producers of propionate in humans.

Butyric acid's structure is a carboxylic acid bonded to two methylene groups bonded to a methyl group (Figure 1) (Butyrate: $CH_3 – CH_2 – CH_2 – COO^-$). In the colon, butyrate is produced by gram-positive Firmicutes, most abundantly *Eubacterium rectale/ Roseburia spp*. and *Faecalibacterium prausnitzii*.[34] The primary pathway in which enteric bacteria produce butyrate is the butyryl-CoA: acetate CoA-transferase pathway (see Figure 1).[28, 31,34] In this pathway butyryl-CoA is produced form Acetyl-CoA. A single enzymatic reaction then converts butyryl-CoA to butyrate.[31] A less common pathway is the butyrate kinase pathway in which phosphotransbutyrylase and butyrate kinase enzymes convert butyryl-CoA into butyrate.[28,31]

SCFA concentration in feces represents ~5% of the total concentration of SCFAs that are produced in the colon, while ~95% of the total SCFAs are rapidly absorbed and utilized by colonocytes or are put into systemic circulation. SCFAs are absorbed in the human colon and cecum at a comparable rate to that of the colonic and cecal mucosa of rats, with a higher rate of uptake seen in the distal colon compared to the proximal colon.[35,36] Ruppin et al perfused the

colon of healthy individuals with an isotonic solution containing 0-90 mM of SCFAs and found that transport of SCFAs across colonocytes into circulation was primarily concentration dependent.[37] The study identified two mechanisms of colonic SCFA absorption: simple diffusion of protonated SCFAs along with consumption of CO2 (~60% of SCFAs), and cellular uptake of ionized SCFAs along with ionic diffusion of sodium and potassium.[35,37,38]

While the absorption of SCFAs is similar, the distribution and fate of SCFAs differ.[28] Oxidation of SCFAs, particularly butyrate, supplies 60-70% of the energy needs of colonocytes (even in the presence of glucose and glutamine which can also serve as substrates for colonocytes).[35] Since butyrate is primarily utilized by epithelial cells, systemic circulation levels of butyrate are relatively low (1-3 mM).[28,39] Propionate is primarily metabolized in the liver and peripheral blood concentrations of propionate are typically low (1-3mM).[28,39] Acetate is the only SCFA to reach high concentrations in peripheral blood (100-150 mM). It is estimated that SCFAs constitute 5-10% of the average person's total metabolizable energy per day.[39]

Physiological Effects

The role of the human microbiome in overall health is a major topic of current research; however, the mechanisms linking the microbiome to health status are largely unknown. Research suggests that a dysbiotic microbiome plays a causative role in several health conditions (e.g. metabolic disorders and inflammatory bowel disease).[40,41] At the same time, a symbiotic microbiome is thought to protect against western diseases.[40,41] It's hypothesized that SCFAs may be the metabolic link between the human microbiome and health status.

Both butyrate and propionate have been shown to inhibit histone deacetylases (HDACs).[42] By inhibiting HDACs, butyrate is able to regulate gene expression and cellular differentiation. This suggests that butyrate may have an anti-carcinogenic effect in colonic

epithelial cells.[28,41,42] Interestingly, butyrate has been shown to increase the proliferation of healthy colonocytes, thereby maintaining/ improving gut integrity; however, in the presence of transformed cells (cancerous colonocytes), butyrate induces terminal differentiation and apoptosis.[43] This is because glucose is the primary fuel source for cancerous colonocytes (in normal colonocytes butyrate is the primary fuel source).[43] As glucose is utilized by colonocytes, butyrate accumulates and functions as a HDAC inhibitor.[43]

Acetate, propionate, and butyrate have also been shown to signal to free fatty acid receptors 2 and 3 (FFAR2, FFAR3), which are G-coupled protein receptors.[39] Karaki et al demonstrated that FFAR2 is expressed in enteroendocrine cells in the ascending colon in humans.[44] Further, activation of FFAR2 by SCFAs has been shown to facilitate/ modify peptide tyrosine tyrosine (PYY) secretion.[39] PYY is a hormone that reduces appetite. Greenway et al. conducted a study to evaluate the effects of fermentable carbohydrates on PYY levels in healthy overweight and obese individuals.[45] After 28 weeks, the researchers saw a significant increase in PYY and satiety 1-hr postprandial compared to baseline measurements ($p<0.01$).[45] This research supports that SCFAs signal to FFAR2 and 3 receptors to increase secretion of PYY in the gut. As a result, increasing SCFA production may play a role in appetite regulation, which can be useful in the management of chronic diseases such as metabolic syndrome and overweight/obesity.

SCFAs also act as mediators of intestinal and systemic immune function by inhibiting HDACs and by activating G-coupled protein receptors of leucocytes and endothelial cells.[46] SCFAs suppress lipopolysaccharide (LPS) and cytokine stimulated production of pro-inflammatory markers (i.e. tumor necrosis factor- alpha, interleukin-6, and nitric oxide). Butyrate, in particular, been shown to enhance the release of interleukin-10, an anti-inflammatory cytokine.[46] Nastasi et al demonstrated that butyrate and propionate exert an

immunomodulatory effect by influencing gene expression in immature and mature human monocyte-derived dendritic cells.[47] In the study, the researchers saw that propionate and butyrate inhibited the expression of LPS induced cytokines (IL-6 and IL-12).[47] Further, the release of pro-inflammatory chemokines (CCL3, CCL4, CCL5, CXCL9, CXCL10, and CXCL11) was significantly reduced following exposure.[47] SCFAs also modify lymphocyte function by inhibiting T-cell proliferation, by reducing the production of pro-inflammatory cytokines, and by producing regulatory T-cells. SCFAs have been shown to improve inflammatory conditions such as inflammatory bowel disease, sepsis, and ischemia induced injury.[45]

Probiotics

Definition and Strains

Probiotics, as defined by the world health organization, are live microorganisms that, when provided in adequate amounts, exert a beneficial effect to the host.[48] Probiotics include several strains of *Lactobacilli* and *Bifidobacteria* as well as *Escherichia coli* strain Nissle 1917, *Saccharomyces boulardii,* and *Streptococcus thermophilus*.[49] Probiotics are naturally found in yogurt, cheese, sauerkraut, kombucha, kefir, and kimchi. Animal and human studies show that probiotics can be supplemented to reduce diarrhea, prevent bacterial infections, manage gastrointestinal diseases, alleviate lactose intolerance and other allergies, and have anti-cancer effects; however, not all probiotic strains can be generalized to have these effects.[50]

When considering the health benefits of probiotic supplements, several factors should be considered. For example, monostrain probiotics (single strain supplements) may have different effects compared to multistrain/multispecies supplements. Timmerman et al evaluated the survivability and activity of a multispecies culture collection containing five strains of *Bifidobacterium (bifidum, breve, infantis, lactis,* and *longum)*, nine strains of *Lactobaccillus*

(*acidophilus, brevis, bulgaricus, casei, helveticus, paracasei, plantarum, rhamnosus,* and *salivarius*), as well as *Enterococcus faecium, Lactococcus lactis,* and *Streptococcus thermophilus*.[51] Timmerman et al found that multispecies probiotics have increased functionality and efficacy in vivo compared to monostrain probiotic cultures.[51] The researchers also found that different strains of probiotics exert different effects. Timmerman et al argue that *B. bifidum, Lc. lactis, L. acidophilus* and *L. casei* are potent immunomodulators while *B. infantis, L. plantarum, L. rhamnosus* and *L. salivarius* exhibit strong antimicrobial activity.[51] That being said, there are currently no guidelines regarding the number of probiotic strains that are optimal for colonization and efficacy. Timmerman et al argues that certain strains, when encapsulated together, exert synergistic effects; however, certain strains, when incorporated into a multi-strain supplement, may inhibit the activity of other strains, thereby reducing the effectiveness of the multi-strain probiotic.[51]

Probiotic strains used in supplements must be stable enough to withstand manufacturing, processing, storage, and transport. Additionally, the efficacy of a probiotic strain is dependent on its survivability in the presence of gastric juice in the stomach as well as enzymes and bile salts in the intestine.[52] Time of probiotic exposure to these factors, degree of stomach acidity, and the concentration of bile salts will also affect viability of probiotics.[52] Research suggests resistance to these factors is strain-dependent.[52] For example, Madureira et al evaluated the survivability and stability of several strains of *B. animalis, L. acidophilus, L. paracasei,* and *L. brevis* when exposed to gastrointestinal conditions.[53] Madureira et al found that all of the strains tested maintained their viability upon exposure to hydrochloric acid and pepsin (gastric juice); however, *L. paracasei ssp.* Paracasei LCS-1 and *B. animalis* BLC-1 experienced a severe decrease in viable cell numbers upon exposure to bile salts.[52,53]

Another significant factor affecting the efficacy of the probiotic supplement is the amount of colony forming units (CFUs) per capsule. The standard probiotic dose thought to be necessary to exert beneficial effects to the host ranges from 10^7 to 10^9 CFUs/mg/day.[54] That being said, processing, storage, and gastrointestinal conditions will alter the CFUs of a given strain, affecting the strain's survivability and overall effectiveness. Research suggests that $>10^6$ CFUs/mL in the small intestine and $>10^8$ CFU/g in the colon is necessary to obtain clinically significant effects.[54] The appropriate probiotic dosage will vary depending on the strain being supplemented, the frequency of supplementation, and the condition for which the probiotic is being supplemented. For example, in the management of acute infectious diarrhea, research suggests that a higher dosage is more effective than a lower dosage; however, higher dosages ($>10^{10}$ CFUs/g) may not be necessary in the management of chronic conditions.[54] Probiotic effects are likely dose-dependent and greater than 10^7 to 10^9 CFUs/mg/day is generally considered the effective dose in humans.[54]

The physiological effects and overall efficacy of a probiotic supplement depends on each of these factors; however, the safety of the probiotic should also be considered. It is generally assumed that probiotics have an "acceptable known safety profile"; however, numerous genera, species, and strains are included under the umbrella term, probiotic, making it difficult to provide a safety assessment statement for all probiotics.[55] Rather, probiotic safety should be determined on a strain-by-strain basis. Well-known and commonly used strains, such as *Lactobaccillus, Bifidobacterium,* and *Saccharomyces* have been determined to be safe for consumption for the general population; however, the manufacturing of the supplement can still impact the product's overall safety.[55] Low quality probiotic supplements should not be used in research and should not be recommended for consumption. Rather, only supplements that are made with Current

Good Manufacturing Practices (CGMP) and products that are Generally Recognized as Safe (GRAS), should be recommended and consumed.[55] As a component of CGMP, probiotic supplements should also meet purity standards and should be manufactured under a quality management system.[55]

Mechanism of Probiotic Action

Probiotics exert health benefits by influencing the composition and function of commensal microbiota, by altering host epithelial and the immune system, and by combating microbial and food toxins that adversely affect human health. That being said, the mechanism by which probiotics exert their 'beneficial effect to the host' is not well understood, this is most likely because probiotics do not have just one mechanism of targeted action. Mechanisms documented in literature include influence on gut barrier function, production of inhibitory substances, immune effects, blockage of adhesion sites, gut microbiota modulation, antiproliferative effects, competition for nutrients, and degradation of toxin receptors (Figure 2).[50]

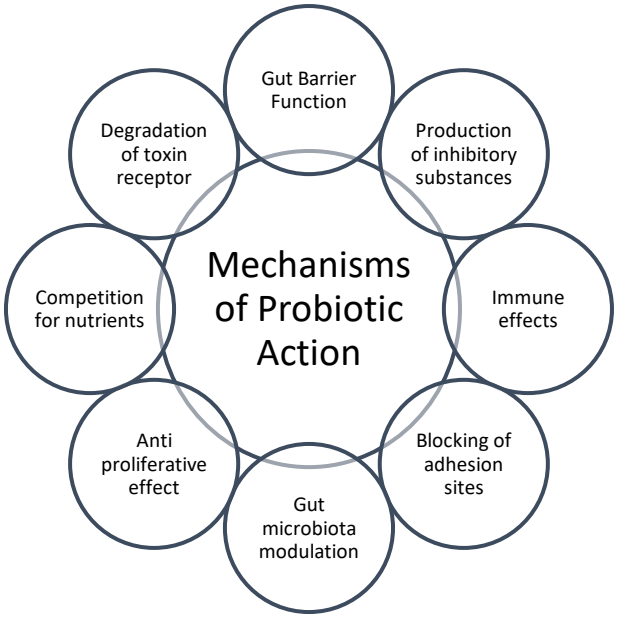


Figure 1: Mechanism of Probiotic Action[50]

One of the main effects of probiotics is colloquially referred to as "killing the bad bacteria." While probiotics accomplish this through several mechanisms, one of the primary mechanisms is the production of antibacterial inhibitory substances, such as bacteriocins, organic acids, and hydrogen peroxide.[50] Bacteriocins are peptides that are active against other bacteria most commonly by inhibiting cell wall synthesis and by destroying target cells by pore formation.[50,56] Different strains of probiotics typically produce different bacteriocins. For example, Corr et al. found that Lactobacillus salivarius UCC118 produces bacteriocin Abp118.[57] Bacteriocin Abp118 has an antagonist effect against Listeria monocytogens and directly protects mice induced with Listeria monocytogens against infection.[57] Bacteriocins produced by lactobacilli and Bifidobacteria have been shown to inhibit several pathogens, including E. coli, Heliobacter pylori, Listeria monocytogens, Rotavirus, and E. coli.[50] Bacteriocins and organic acids such as lactic acid and SCFAs (byproducts of probiotics) also lower the pH within the GI tract. While lactic acid producing bacteria such as lactobacilli and Bifidobacteria can survive well in an acidic environment, many pathogenic bacteria cannot.

Another mechanism of probiotic efficacy is the ability to block adhesion sites for pathogen bacteria.[50] Probiotic strains compete for adhesion sites along the epithelial surface of the gastrointestinal tract, inhibiting the colonization of pathogenic bacteria, causing pathogens to pass through the GI tract for excretion. Acid-resistant probiotics (i.e. Bifidobacterium longum and Bifidobacterium catenulatum) have demonstrated better adhesion to the epithelial surface compared to acid-sensitive strains. Additionally, multi-strain probiotics (i.e. VSL#3) can modulate gene expression and improve the adhesion of bacterial cells to the GI tract.
Health promoting bacteria also compete more efficiently for nutrients, which prevents pathogen's growth and proliferation. For example, while iron is an essential nutrient needed for

the survival of most bacteria, it is not needed for lactobacilli bacteria. Additionally, certain strains of Lactobacilli, such as L. acidophilus and L. delbrueckii, can bind ferric hydroxide at their cell surface, making iron less available for pathogens. This essentially creates an environment in which certain pathogens cannot survive.[50]

Probiotics may also have anti-proliferative effects, as indicated in recent research. Certain probiotics are able to metabolically inactivate mutagenic substances, while others can bind heterocyclic aromatic amines and N-nitroso compounds to reduce DNA damage and levels of cancer-causing compounds.[50] Additionally, probiotics influence on cytokine production and T-cell function may promote anti-tumor activity by amplifying the immune response of tumor tissue.[50] Research also shows that enhanced levels or lactobacilli and Bifidobacteria and lower amounts of Clostridium, coliforms, and Bacteroides are associated with reduced incidence of colorectal cancer.[50]

Therapeutic Effects of Probiotics on GI Upset

Probiotics likely have an indirect influence on SCFAs by enriching specific gut microbial populations that preferentially ferment carbohydrates.[49] Meimandipour et al. found that supplementation of *Lactobacillus agilis* and *Lactobacillus salivarius* significantly increased production of lactate, propionate, and butyrate in vitro in cecal microflora of chickens.[58] Another in vitro study conducted by Ogawa et al. showed that specific Lactobacillus species (*L. casei* and *L. acidophilus*) inhibited growth and activity of Shiga toxin-producing Escherichia coli O157:H7, a pathogenic bacterium.[59] In vitro studies suggest that probiotics exert a positive effect on the microbiome by promoting the colonization of beneficial bacteria and by inhibiting the growth of opportunistic pathogens, both of which have an indirect effect on SCFA production. In vitro studies are beginning to be supported by human randomized controlled trials (RCT). In an

RCT conducted by Wang et al. the researchers supplemented 6.0 x 10^{10} CFUs/day of *Lactobacillus plantarum* to healthy individuals for four weeks.[60] Fecal concentrations of Bifidobacterium significantly increased ($p<0.05$), concentrations of Desulfovibrio (an opportunistic pathogen) significantly decreased ($p<0.05$), and acetate and propionate concentrations significantly increased ($p<0.05$) after 4 weeks of supplementation.[60]

Prebiotics

Definition and Sources

A prebiotic is a food ingredient not digested in the upper GI tract that selectively stimulates the growth and/or activity of one or a limited number of bacteria in the colon.[49] Prebiotics also reduce non-beneficial (pathogenic) bacterial populations. Prebiotics include inulin, fructo-oligosaccharides, and resistant starch and are naturally found in foods such as beans, peas, oats, onions, garlic, tomatoes, and bananas. Fibers that act as prebiotics include cellulose, lignin, hemicellulose, pectins, and fructans (i.e. fructo-oligosaccharides and inulin).

Therapeutic Effect of Prebiotics on GI Upset

Numerous studies have documented the potential of prebiotics to increase SCFA production. Cardelle-Cobas et al demonstrated that galacto-oligosaccharides (GOS) stimulated Bifidobacteria growth and increased production of acetic acid in human fecal cultures (*in vitro*).[61] In a similar study using fecal inocula incubation, Hernot et al found that short-chain oligosaccharides (e.g. NutraFlora, GTC Nutrition, Golden) were more rapidly fermented compared to long-chain oligosaccharides (e.g. inulin- Beneo ST and HP).[62] *In vitro*, Lactobacilli concentrations increased similarly amongst short and long-chain oligosaccharides; however, Bifidobacteria concentrations significantly increased when short-chain oligosaccharides were fermented.[62] The increase in Bifidobacteria was accompanied by an increase in gas, acetate and

propionate production in short-chain oligosaccharides.[62] In a pilot human study conducted by Schwiertz et al, supplementing 15 grams of resistant starch per day for 28 days resulted in a significant increase fecal butyrate concentrations; however, total SCFA concentration remain unchanged.[63] The research conducted by Cardelle-Cobas, Hernot, and Schwiertz suggest that prebiotics directly impact SCFA production.[61,62,63] Further, prebiotic chain length influences fermentation rates, gas production, and microbial profiles.[49]

Synbiotics

Synbiotics are combination probiotic and prebiotic supplements. Many researchers hypothesize that synbiotics enhance colonic fermentation and promote gut symbiosis at a greater level than probiotics or prebiotic supplements that are administered alone.[49,64] Grela et al conducted a study to evaluate the effects of multi-strain probiotics alone (*Lactococcus lactis, Carnobacterium divergens, Lactobacillus casei, Lactobaccillus plantarum,* and *Saccharomyces cerevisiae*), prebiotics alone (inulin), or both probiotics and prebiotics on select markers of the gastrointestinal system, including SCFA concentrations, in pigs.[65] The concentration of propionic, butyric, and valeric acids increased significantly in the prebiotic group and in the prebiotic plus probiotic group when compared to a control group ($p<0.05$).[65] A similar study was conducted in healthy individuals by Worthley et al (34). The researchers did not observe a significant change in SCFA concentrations in the probiotic group (*Bifidobacterium lactis*), the prebiotic group (high-amylose maize starch), or the synbiotic group (probiotic + prebiotic) after 4 weeks.[64] More research is needed to understand the effects of synbiotics on SCFA concentration in humans based on the above study and a general lack of available human research on synbiotics. That being said, results from studies on prebiotic and probiotic supplementation alone support the use of probiotics and prebiotics for the modulation of SCFAs.

Enzymes

Digestive enzymes include proteases, peptidases, lipases, amylases, nucleases, cellulases, and lactase. Digestive enzymes speed up and improve the breakdown of macromolecules (fats, carbohydrates, and proteins) into smaller molecules that are more easily absorbed.[66] In addition to probiotics and prebiotics, digestive enzymes may improve gastrointestinal symptoms and may modulate the gut microbiome. Spagnuolo et al evaluated the effects of combination β-glucan, inositol, and digestive enzyme supplementation on gastrointestinal symptoms of individuals with IBD and IBS.[66] The researchers found a significant reduction in the intensity of abdominal pain ($p<0.01$), bloating ($p<0.001$), and flatulence ($p<0.005$) over 4 weeks; however the group did not experience any significant changes in inflammatory markers during the study.[66] In another study conducted by Quinten et al, individuals with common gastrointestinal problems randomly received either domperidone (n=19) or Similase total (n=43).[67] Similase total is an enzyme supplement that contains protease, cellulase, amylase, alpha galactosidase, maltase, lactase, lipase, invertase, and phytase, while domperidone (Motilium) is a common antiemetic and prokinetic medication for gastrointestinal disorders.[68] Quinten et al found that participants receiving Similase total experienced a more significant reduction in abdominal pain compared to those receiving domperidone after 5 days of treatment ($p=0.021$).[67]

In addition to improving gastrointestinal symptoms, digestive enzymes may influence the gut microbiome, however research is limited and nascent in this area. Nishiyama et al divided mice into two groups- the control, who received tap water, and the treatment group, who received lipase, amylase, and protease.[69] The mice that received the enzyme treatment had a significantly different microbiota after 21 days.[69] Specifically, treated mice had a higher

abundance of *Akkermansia muciniphila* and *Lactobacillus reuteri*, two beneficial commensal bacteria, in the cecum.[69]

CHAPTER 3

MATERIALS AND METHODS

Participant Selection

Potential participants responded to a flyer advertised at East Tennessee State University sent through a mass email to students, faculty, and staff, or through advertisement at Johnson City health food stores, Johnson City community/ senior centers, and other retail establishments (See Appendix A). Potential participants were then screened for eligibility using a phone interview (See Appendix B). Participants met the following criteria of inclusion (1) self-reported presence of GI symptoms greater than 3 times per week (i.e. abdominal pain, heartburn, acid regurgitation, bloating, nausea and vomiting, abdominal distension, burping, increased gas, decreased passage of stools, increased passage of stools, loose stools, hard stools, urgent need for defecation, feeling of incomplete evacuation), (2) self-identified as healthy or having few health complications, and (3) over the age of 18 years old. Criteria of exclusion included (1) under the age of 18 years old, (2) diagnosis of Celiac disease, irritable bowel syndrome, inflammatory bowel disease (including Crohn's disease and Ulcerative Colitis), (3) previous use/ consumption of GlutenShield, (4) current consumption of any prebiotics, probiotics, enzymes, non-steroidal anti-inflammatory drugs (NSAIDS), fish oil, and/or fiber supplements unless the participant was willing to halt use 2 weeks prior to beginning the study), (5) Recommendation/ prescription use of any NSAID, and (6) pregnancy or intention to become pregnant within 60 days.

Study Design

The study was a split, 28-day randomized, partially blinded design. Eligible participants were administered an informed consent document by the principal investigator. After completing the informed consent, subjects were randomly assigned to one of two groups using a computer random number generator. Group A completed a 2-week washout of any probiotics, prebiotics, enzymes, NSAIDS, fish oil, and fiber supplements followed by a 28-day treatment period with GlutenShield taken 3x/day with meals. Group B completed the same 2-week washout followed by a 28-day placebo period with the placebo taken 3x/day with meals. Participants were blinded to which group/ treatment they were given. Subjects were provided a procedure form and materials to collect a stool sample at home when meeting to fill out the informed consent.

On day zero of the active study, subjects arrived at Hutcheson Hall at ETSU and submitted a stool sample to the principal investigator. At this time, Dr. Kenneth Phillips collected two tubes of whole blood. Participants also completed a food frequency questionnaire, gastrointestinal symptoms questionnaire, and mood assessment on day zero. Participants were provided either a bottle of GlutenShield or a bottle of placebo pills. Participants were then instructed to take 3 pills per day with meals beginning on day 1 and lasting through day 28. On day 28, participants came back to Hutcheson Hall at ETSU and submitted a second stool sample to the principal investigator while Dr. Phillips again collected two tubes of whole blood. Participants also completed the gastrointestinal symptoms questionnaire and the mood assessment on day 28. Full review approval was obtained for this study by the ETSU Institutional Review Board (IRB) on December 5, 2017; study number 1117.22f.

Placebo and GlutenShield

The placebo for the study was prepared by Dr. Charles Collins in the ETSU college of pharmacy. Each placebo capsule contained a 50/50 mixture of Avicel (cellulose) and of bentonite powder (to have a similar color as GlutenShield). The placebo was encapsulated in Vcaps Enteric and were made of cellulose.

GlutenShield is a combination probiotic, prebiotic, and enzyme dietary supplement. Probiotics in GlutenShield include *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Bifidobacterium lactis*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus salivarius*, *Streptococcus thermophilus*, *Bifidobacterium bifidum*, *Lactobacillus coagulans*, and *Saccharomyces boulardii*. Prebiotics in GlutenShield include Chitosan oligosaccharide, fructooligosaccharides, alfalfa, Emblica officinalis extract, papaya juice powder, fulvic acid, and ionic minerals. Enzymes in GlutenShield include dipeptyl peptidase IV (DPPV-IV), lactase, cellulase, hemicellulase, xylanase, phytase, serrapeptase, and plant-based digestive enzymes (lipase, protease, and amylase).

Assessments

Demographics and Food Frequency Questionnaire

On day 0 of the active trial, study participants completed NutritionQuest's 2014 Block Food and Activity Questionnaire, a food frequency questionnaire (FFQ). The form measured usual dietary intake over the past month. Participants were instructed to report how often they consume a given food (i.e. cold cereal- once a month, 2-3 times per month, once per week, 2 times per week, 3-4 times per week, 5-6 times per week, or every day), how much of the given food they consume in a given day (i.e. 1 glass of orange juice, 1 cup of cold cereal), and what

type of food they consume (i.e. low-fat, sugar-free). The questionnaire provided a detailed look at typical consumption of:

- Eggs and dairy foods
- Cereals, grains, and breads
- Vegetables
- Fruits
- Beans, tofu, and meat substitutes
- Soups, mixed dishes, and noodles
- Meat and chicken
- Fish and seafood
- Nuts, seeds, and snacks
- Sweets and desserts
- Spreads, sauces, and other foods
- Beverages

From the self-reported intake, macronutrient and micronutrient intake was determined. In addition to reported typical food intake, respondents self-reported sex, age, weight, and height, from which body mass index (BMI) was calculated. Consumption of multiple and single vitamins and minerals as well as typical physical activity and ethnicity were also recorded.

Gastrointestinal Symptoms Questionnaire

Participants completed the GI symptoms questionnaire on day 0, prior to the intervention, and on day 29, after the intervention. The questionnaire measured the severity of GI symptoms using a Likert numerical scale rating ranging from 1 (none) to 7 (always/severe) over the past week. GI symptoms measured included abdominal pain/ discomfort, heartburn, acid

regurgitation, bloating, nausea and vomiting, abdominal distension, eructation (burping), increased gas, decreased passage of stools, increased passage of stools (rapid transit), loose stools, hard stools, urgent need for defecation, and feeling of incomplete evacuation. The questionnaire was based off of Catassi et al's Solerno experts diagnostic criteria.[2] The questionnaire was administered in paper-and-pencil formation and took 3-5 minutes to complete.

POMS Assessment

Participants completed MHS's Profile of Mood States Second Edition adult short assessment (POMS 2-A-S) on day 0, prior to the intervention, and on day 29, after the intervention. The POMS short assessment was designed for adults ages 13 to 50+ and contained a subset of 35 items taken from the full-length assessment. The assessment provided a cursory evaluation of scaled scores for anger/ hostility, confusion/ bewilderment, depression/ dejection, fatigue/ inertia, tension/ anxiety, vigor/ activity, and friendliness. The assessment was used to evaluation changes in total mood disturbance at a given moment prior-to and following the intervention. The assessment was self-administered in paper-and-pencil format and took 3-5 minutes to complete. Scores were measured using a t-score for each category that were automatically calculated by MHS Online Assessment Center. A t-score of >70 indicated a very elevated score, 60-69 indicated an elevated score, 40-59 indicated an average score, 30-39 indicated a low score, and <30 indicated a very low score (see Table 2).

Table 2: POMS T-Score Classifications[70]

T-Score	Classification	
	Negative Mood State	**Positive Mood State**
70+	Very Elevated Score (many more concerns than are typically reported)	Very Elevated Score (Far fewer concerns than are typically reported)
60-69	Elevated Score (more concerns that are typically reported)	Elevated Score (Fewer concerns than are typically reported)
40-59	Average Score (Typical levels of concern)	Average Score (Typical levels of concern)
30-39	Low Score (Fewer concerns than are typically reported)	Low Score (More concerns than are typically reported)
<30	Very Low Score (Far fewer concerns than are typically reported)	Very Low Score (Many more concerns than are typically reported)

Blood Collection and Analysis

Dr. Kenneth Phillips, associate dean of research for ETSU's college of nursing collected 10 mL of whole blood using a 23-gauge butterfly needle into two 8 ½ mL red-with-black top Becton Dickinson vacutainers per participant on day 0, prior to the intervention, and on day 29, post-intervention. The samples were put on ice and transported to ETSU's health science laboratory were allowed to clot at room temperature for 30 minutes. The clot was removed by centrifuging the samples at 3000 x g for 10 minutes. One mL of the supernatant (serum) was transferred into a 1.5mL polypropylene Fisherbrand micro-centrifuge tube using am Eppendorf pipette. Two mL of serum was transferred to a 2.7 mL Fisher Scientific amber vial. The samples were stored at -80° Celsius for future analysis. ELISA analysis was performed using commercially available ELISA plates from Aviscera Bioscience. The remaining sample (2.7 mL) was banked for future analysis.

Fecal Collection

Following the informed consent meeting, participants were given a stool collection procedure form and were told how to collect a stool sample (See Appendix C). Participants were provided with saran (plastic) wrap, 4 self-sealing plastic bags, and 2 pairs of large nitrile gloves. Participants were instructed to collected 2 stool samples- one on day 0, prior to the intervention, and one on day 29, following the intervention. Participants collected the stool sample by lifting the toilet seat and placing a layer of saran (plastic) wrap across the toilet, leaving a dip in the middle. The bowel movement was then wrapped in the saran wrap, placed in a self-sealing plastic bag, and frozen in the freezer. Participants then transported the stool sample to Hutcheson Hall at ETSU on day 0 and day 29. Participants collected and froze the stool sample within 24-hours of transporting the sample to ETSU. The stool samples were then stored on ice in a biohazard cooler and were transported to the Human Nutrition and Dietetics Research Laboratory on ETSU's Valleybrook campus. At the laboratory, approximately 1 gram of fresh sample was separated and stored at -80° Celsius for future PCR microbiome analysis. The remaining sample was freeze-dried and used for nutrient analysis.

Fecal Analysis

Freeze Drying

The stool samples were freeze-dried using the FreeZone 2.5 Liter Freeze Dryer System. The instrument and refrigeration switch were turned on and allowed to reach -40° Celsius. Once -40°C was reached, the vacuum was switched on and the instrument was ready for use. A 600 mL LABCONCO freeze dry flask and lid was weighed and the tare weight was recorded. The pre-frozen stool sample was placed in the freeze-dry flask and the lid was sealed tightly. The flask, lid, and sample were weighed and the weight was recorded. The stool sample weight (wet

weight) was then calculated and recorded. The sample was place on the freeze-dryer and was dried for ~48 hours at 0.077 mBar and -50° Celsius until all frost and cold spots were gone and the sample was thoroughly dry. The sample was removed from the freeze dryer and the flask, lid, and sample were weighed and the weight was recorded. The sample weight after freeze-drying (dry weight) and the percent dry weight were then calculated. Samples were then ground to a fine powder using an IKA M20 Universal Mill.

Table 3: Freeze-Drying Calculations

Freeze-Drying Calculations	
Wet Weight	(flask + lid + sample before) – (flask + lid)
Dry Weight	(flask + lid + sample after) – (flask + lid)
Percent Dry Matter	Dry weight / Wet weight x 100

Kjeldahl Digestion

Total nitrogen was determined for freeze-dried and ground samples using kjeldahl digestion. For the procedure, 100 mg of the sample was weighed (weight was recorded) into a 100 mL kjeldahl flask along with 1.9 grams of potassium sulfate (K_2SO_4), 80 mg of mercuric oxide (HgO), 2 mL of concentrated (10N) sulfuric acid, and 2 porous boiling chips. The sample was placed on LABCONCO heat mantle. The air was turned on and the mantle was turned to heat setting 3. The sample refluxed for 8-12 hours, the heat mantle was then turned off, and the sample was cooled to room temperature. 15 mL of deionized distilled water (DDW) was added to the kjeldahl flask. The sample was brought to a boil and was filtered while hot into a 150 mL Erlenmeyer flask using P5 grade Fisher brand qualitative grade plain filter paper circles. Following digestion of the samples, distillation was performed to determine total nitrogen per sample. The LABCONCO rapid distillation unit was turned on, set to heat setting of 6-7, and was allowed to heat up. 5mL of 4% Boric acid and a few drops of kjeldahl indicator were added

to a new 150 mL Erlenmeyer flask. The flask was placed at the bottom of the distillation unit. The distillate (filtered sample) was added to the top of the unit and the Erlenmeyer flask was rinsed with DDW. The material was emptied into the reaction tube. 10 mL of sodium thiosulfate (NaOH/ $Na_2O_3S_2$) was added to the top of the apparatus and was slowly emptied into the reaction tube. The sample was allowed to distill for 15-20 minutes until the total volume of the boric acid and ammonium solution reached 25-30 mL. During the distillation process, ammonium (NH_4^+) was converted to ammonia gas (NH_3^+). NH_3^+ condensed into the boric acid solution to form ammonium borate. The ammonium borate was then titrated with 0.1 N HCl until a color change was observed (blue→red; base→acid). The mL of HCl needed to titrate the solution back to an acid was recorded. Nitrogen per kilogram of sample and percent total protein were then calculated. Samples were run in duplicate.

Table 4: Kjeldahl Calculations

Kjeldahl Calculations	
Grams of Nitrogen per Kilogram of Sample	(mL HCl titrated x 0.1N x 14.01) / weight of sample (grams)
Percent Total Protein	(g N per kg) x 6.25 / 1000g x 100

Total, Soluble, and Insoluble Dietary Fiber Analysis

Total dietary fiber (TDF), soluble dietary fiber (SDF), and insoluble dietary fiber (IDF) was assessed on freeze-dried, ground stool samples using the automated ANKOM Dietary Fiber Analyzer method AOAC 991.43.[72] Reagents were prepared as shown in Table 5.

Table 5: Fiber Reagent Methodology[72]

Reagent	Method
78% ETOH	Dilute 410.5 mL of 95% ETOH to 500 mL using DDW water in a 500 mL volumetric flask.
Enzyme Solutions - α-Amylase - Protease - Amyloglucosidase	 Dilute 5 mL of α-Amylase to 25 mL using DDW Dilute 5 mL of Protease to 25 mL using DDW Dilute 5 mL of Amyloglucosidase to 25 mL using DDW
MES-TRIS Buffer	Dissolve 9.76 g of 2-(N-Morpholino)ethansulfonic acid (MES) and 6.1 g of Tris(hydroxymethyl)aminomethane (TRIS) in 850 mL of DDW. Adjust pH to 8.2 using 6*N* NaOH and dilute to 1 L with DDW
Dilute HCl Solution (0.561*N*)	Dilute 23.375 mL of 6*N* HCl to 250mL in a volumetric flask using DDW.

ANKOM IDF and SDF filter bags were labeled with a permanent marker. Each bag was weighed using the Bag Weigh Holder and an AL54 Mettler Toledo analytical balance. The tare bag weight was recorded onto a Dietary Fiber Data Spreadsheet (DFDS). One gram of Diatomaceous Earth was weighed into two separate dishes. The weight of each was recorded onto the DFDS. 0.5±0.05 g of the freeze dried, ground stool sample was weighed in duplicate into two dishes. The weight of each was recorded onto the DFDS. All fluid levels were checked on the DF analyzer. The instrument and Nitrogen gas were turned on. SDF bags and Clamp Bar D were installed on the instrument. Clamp Bar D was closed and the pre-weighed DE was added to each SDF bag and was rinsed with 2-3mL of DI water. IDF bags and Clamp Bars B and C were installed onto the instrument. Clamp Bar B was closed, pinching off the bags, and the pre-weighed samples were transferred to the IDF bags. SDF bags were hooked to Clamp Bar C. Clamp Bar A was installed. The instrument was started, beginning the automated process of digesting the sample. After the amylase and protease phases, the pH of the samples was checked

and adjusted to 4.0-4.7 as needed with 0.561*N* HCl. After the automated process was complete, IDF and SDF bags were rinsed with Acetone using the ANKOM Acetone Rinse Stand. After drying, the bags were sealed at a heat setting of 3 using the ANKOM Heat Sealer. Samples were placed on a drying rack and were placed in a Fisher Scientific Isotemp oven at 100° Celsius for 90 minutes. Samples were removed from the oven and immediately placed in an ANKOM *MoistureStop* weigh pouch (desiccant pouch) to cool. Bags were removed one at a time from the desiccant pouch and were weighed on an analytical scale using the Bag Weigh Holder. Bag weights were recorded on the DFDS. A protein correction was performed using kjeldahl digestion and distillation, as described above. An ash correction was also performed by burning the samples as 700° Celsius for 5 hours and recording the weight after ashing. All ashing and protein values were recorded on the DFDS. Percent IDF, SDF and TDF were then calculated using the following equations:

- %IDF or %SDF = (Residue (grams) – protein residue and bag – ash residue and bag – blank) / original weight of sample x 100%
- TDF = %IDF + %SDF

SCFA Extraction and Determination

SCFA extractions were performed using a procedure developed by Schwiertz et al. that was modified.[73] One mL of the SCFA extraction solution, containing Oxalic acid (0.1 mol/L), Sodium Azide (40 mmol/L), and Caproic acid (0.1 mmol/L)(internal standard) was added to 80 mg of a freeze-dried stool sample in a 16 x 100 mm disposable culture tube. The tube was capped and vortexed for 30 seconds. The tube was placed on a horizontal shaker for 1 hour. The tube was centrifuged at 4000 rpm for 20 minutes. After centrifuging, the supernatant was removed and placed in a 1.5mL polypropylene Fisherbrand micro-centrifuge tube. The solution

was re-centrifuged at 12,000 rpm for 15 minutes. Again, the supernatant was removed and placed in a new 1.5 mL micro-centrifuge tube. The solution was re-centrifuged at 12,000 rpm for 15 minutes. Finally, the supernatant was removed, placed in a 2 mL amber vial, and was stored at -80° Celsius until being analyzed using a Shimadzu GC2010 gas chromatograph with Sigma-Aldrich ZB-Wax Plus capillary column. Samples were run using the SCFA.standard.run.gcm method adapted from Schaefer et al. shown in Table 6.[74]

Table 6: SCFA Gas Chromatography Methodology[74]

<table>
<tr><th colspan="2">SCFA.standard.run.gcm Method</th></tr>
<tr><td>Injection Volume</td><td>1 μL</td></tr>
<tr><td>SPL1 Temperature</td><td>750° Celsius</td></tr>
<tr><td>Column Parameters:</td><td>Initial temperature: 50° Celsius
<table>
<tr><th>Rate</th><th>Temperature (° C)</th><th>Hold (minutes)</th></tr>
<tr><td>-</td><td>50</td><td>2</td></tr>
<tr><td>15</td><td>140</td><td>5</td></tr>
<tr><td>10</td><td>160</td><td>3</td></tr>
<tr><td>10</td><td>175</td><td>3</td></tr>
</table></td></tr>
<tr><td>FID1 Temperature</td><td>180° Celsius</td></tr>
<tr><td>End Time</td><td>24 minutes</td></tr>
</table>

Statistical Analysis

Descriptive statistics and subsequent analyses were performed using GraphPad Prism. General linear models were used for all statistical analysis to determine change over time within the treatment and placebo groups and to determine differences between treatment and placebo participants. Change over time within a group and differences between groups were considered significantly significant if a p-value of less than 0.05 was achieved. Standard deviations were also determined for all dependent variables.

CHAPTER 4

RESULTS

Participants

Twenty-two participants were enrolled in the study per eligibility criteria. Two participants were removed from the study, and 20 participants completed the study. One participant was removed for an injury unrelated to the study that prevented further continuation. The other was removed for experiencing increased gastrointestinal symptoms while taking the placebo. Of the 20 that completed the study, group one included 10 participants that took the treatment (GlutenShield) three times a day for 28 days and group two included 10 participants that took the placebo three times a day for 28 days.

Demographics

The overall cohort (n=20) had a mean age of 42 ± 17.11, with a mean age of 46.80 ± 17.25 for the treatment group and a mean age of 37.2 ± 16.42 for the placebo group. The total cohort was comprised on 30% males (n=6) and 70% females (n=14) with 3 males and 7 females in both the treatment and placebo groups. The mean BMI for the overall cohort was 28.61 ± 7.26, with a mean BMI of 24.79 ± 6.42 for the treatment group and a mean BMI of 31.49 ± 7.21 for the placebo group. The treatment group was comprised of 60% normal weight (NW) adults (n=6), 30% overweight (OW) adults (n=3), and 10% obese (OB) adults (n=1) using BMI classification. The placebo group was comprised on 20% NW adults (n=2), 30% OW adults (n=3), and 50% OB adults (n=5). In the overall cohort, 90% (n=18) identified as Caucasian, while 10% (n=2) identified as other. Demographic results are shown in Table 7.

Table 7: Demographics

	Overall Cohort	Treatment Group	Placebo Group	p*
N	20	10	10	
Gender				
Male gender n (%)	6 (30%)	3 (30%)	3 (30%)	1.000
Female gender n (%)	14 (70%)	7 (70%)	7 (70%)	1.000
BMI				
BMI (kg/m2)	28.61 ± 7.26	24.79 ± 6.42	31.49 ± 7.21	0.078
-Normal Classification (%)	8 (40%)	6 (60%)	2 (20%)	0.074
-Overweight Classification (%)	6 (30%)	3 (30%)	3 (30%)	1.000
-Obese Classification (%)	6 (30%)	1 (10%)	5 (50%)	0.054
Ethnicity				
Caucasian	18 (90%)	9 (90%)	9 (90%)	1.000
Other	2 (10%)	1 (10%)	1 (10%)	1.000
Age (years)	42.0 ± 17.11	46.80 ± 17.25	37.2 ± 16.42	0.219

Assessments

Food Frequency Questionnaire and Physical Activity

Results from NutritionQuest's 2014 Block Food and activity questionnaire are shown in Tables 8 through 12. Meal frequency for the cohort (n=20) averaged 2.63 ± 0.60 and snack frequency averaged 1.89 ± 0.90 (see Table 8). There were no significant differences in macronutrient distribution between groups ($p>0.05$). For the total cohort, daily energy intake averaged 1543.41 ± 517.95 calories, protein intake averaged 60.26 ± 23.83 grams (15.62% of total caloric intake), carbohydrate intake averaged 171.72 ± 62.29 grams (44.50% of total caloric intake), total fat intake averaged 67.65 ± 27.92 grams (39.46% of total caloric intake), fiber intake averaged 15.22 ± 7.45 grams, insoluble fiber intake averaged 10.86 ± 6.17 grams, and soluble fiber intake averaged 4.02 ± 1.73 grams. A more detailed breakdown of macronutrient intakes in shown in Table 9.

Table 8: FFQ: Meal/Snack Frequency

Meal/Snack Frequency				
Item (per day)	**Treatment Group (mean ± std.)**	**Placebo group (mean ± std.)**	**Total Cohort (mean ± std.)**	**p***
Meal frequency	2.6 ± 0.52	2.67 ± 0.71	2.63 ± 0.60	0.816
Snack frequency	1.8 ± 0.79	2.00 ± 1.07	1.89 ± 0.90	0.654

Table 9: FFQ: Macronutrient/ Other Intake

Macronutrient/ Other				
Item (per day)	**Treatment Group (mean ± std.)**	**Placebo group (mean ± std.)**	**Total Cohort (mean ± std.)**	**p***
Energy, total (kcals)	1609.40 ± 463.41	1447.41 ± 584.75	1543.41 ± 517.95	0.583
Protein (g)	64.32 ± 21.67	56.20 ± 26.31	60.26 ± 23.83	0.461
Carbohydrates (g)	178.98 ±50.44	164.45 ± 74.36	171.72 ± 62.29	0.615
Total fat (g)	69.32 ± 29.97	65.99 ± 27.23	67.65 ± 27.92	0.797
Saturated fat (g)	21.02 ± 8.49	22.08 ± 9.78	21.54 ± 8.93	0.799
Monounsaturated fat (g)	26.07 ± 11.20	24.97 ± 10.22	25.52 ± 10.45	0.822
Polyunsaturated fat (g)	16.45 ± 9.09	13.34 ± 5.48	14.92 ± 7.48	0.358
Cholesterol (mg)	211.86 ± 93.93	218.23 ± 101.85	215.08 ± 95.41	0.885
Alcohol (g)	5.73 ± 6.31	4.47 ± 5.06	5.10 ± 5.60	0.630
Total sugar (g)	72.59 ± 35.47	75.93 ± 43.82	74.26 ± 38.84	0.853
Total fiber (g)	16.69 ± 6.97	13.75 ± 7.99	15.22 ± 7.45	0.392
Insoluble fiber (g)	12.04 ± 5.92	9.67 ± 6.49	10.86 ± 6.17	0.403
Soluble fiber (g)	4.55 ± 1.91	3.48 ± 1.43	4.02 ± 1.73	0.175

Intake of grains, whole grains, vegetables, fruit, dairy, protein, and beneficial oil intake is also shown in Table 10. Results are reported in equivalents shown in relation to the United States Department of Agriculture's Dietary Guidelines for Americans/ MyPlate recommendations. Grain intake averaged 4.04 ± 2.34 oz per day, whole grain intake averaged 0.92 ± 0.58 oz per day, vegetable intake averaged 1.06 ± 0.63 cups per day, fruit intake averaged 0.73 ± 0.75 cups per day, dairy intake averaged 1.26 ± 0.65 cups per day, protein intake averaged 5.2 ± 3.88 oz per day, and beneficial oil intake averaged 5.11 ± 2.67 teaspoons per day. All p-values for measurement between groups for MyPlate equivalents were greater than 0.05. When comparing intakes of food groups to the MyPlate recommendations, the total cohort averaged a lower intake of grains (average intake (AI) : 4.04 ± 2.34, recommended intake (RI): 5 oz equivalent), whole grains (AI: 0.92 ± 0.58, RI: 3 oz-equivalent), fruit (AI: 0.73 ± 0.75, RI: 1.5 cup equivalent), dairy (AI: 1.26 ± 0.65, RI: 2.5 cup equivalent), and beneficial oils (AI: 5.11 ± 2.67, RI: 17 grams) based on a 1,400 calorie meal pattern.[75]

Table 10: FFQ: MyPlate Equivalents

MyPlate Equivalents				
Item (per day)	**Treatment Group (mean ± std.)**	**Placebo group (mean ± std.)**	**Total Cohort (mean ± std.)**	**P***
Grain (oz)	4.34 ± 1.63	3.74 ± 3.0	4.04 ± 2.34	0.512
Whole grain (oz)	1.16 ± 0.65	0.67 ± 0.40	0.92 ± 0.58	0.060*
Vegetable, total (no legume or potato) (cup)	1.30 ± 0.71	0.83 ± 0.47	1.06 ± 0.63	0.100
Vegetable, potato and legume (cup)	0.29 ± 0.22	0.20 ± 0.12	0.25 ± 0.18	0.265
Fruit, total (including juice) (cup)	0.53 ± 0.38	0.93 ± 0.98	0.73 ± 0.75	0.247
Dairy, total (cup)	1.23 ± 0.60	1.29 ± 0.73	1.26 ± 0.65	0.847
Protein foods: meat, poultry, seafood (oz)	3.23 ± 1.68	3.12 ± 1.89	3.17 ± 1.74	0.894
Protein foods: nuts and seeds (oz)	1.34 ± 1.60	0.66 ± 0.52	1.00 ± 1.21	0.217
Protein foods: eggs (oz)	0.40 ± 0.23	0.42 ± 0.32	0.41 ± 0.27	0.875
Protein foods: legumes and soy (oz)	0.92 ± 0.85	0.33 ± 0.12	0.62 ± 0.66	0.042*
Beneficial oils (tsp)	5.63 ± 3.27	4.59 ± 1.94	5.11 ± 2.67	0.398

Micronutrient distribution of daily vitamin and mineral intake is shown in Table 11. No significant differences were indicated between groups for micronutrient intake ($p>0.05$). Physical activity results are shown in Table 12. Overall daily estimated energy expenditure for all activities averaged 730.09 ± 565.16 calories and estimated activity expenditure excluding work and chores averaged 206.65 ± 245.84 calories per day. Minutes spent performing activities per day averaged 89.79 ± 66.42 for light activity, 46.51 ± 67.86 for moderate activity, and 18.44 ± 23.59 for vigorous activity.

Table 11: FFQ: Micronutrient Intake

Micronutrient Intake				
Item (per day)	**Treatment Group (mean ± std.)**	**Placebo group (mean ± std.)**	**Total Cohort (mean ± std.)**	**P***
Vitamin A (mg)	631.70 ± 251.54	494.81 ± 158.12	563.25 ± 216.20	0.162
Vitamin E (mg)	8.25 ± 3.53	6.19 ± 1.82	7.22 ± 2.93	0.118
Vitamin E, added (mg)	0.56 ± 0.90	0.26 ± 0.20	0.41 ± 0.65	0.311
Vitamin D (mcg)	4.06 ± 1.97	2.93 ± 1.22	3.49 ± 1.70	0.141
Vitamin K (mcg)	128.82 ± 75.79	82.30 ± 36.54	105.56 ± 62.63	0.097
Vitamin C (mg)	53.12 ± 25.33	56.22 ± 30.04	54.67 ± 27.09	0.806
Thiamin (mg)	1.90 ± 1.07	1.58 ± 0.76	1.74 ± 0.92	0.451
Riboflavin (mg)	1.27 ± 0.39	1.04 ± 0.50	1.15 ± 0.45	0.265
Niacin (mg)	21.41 ± 9.22	19.01 ± 9.43	20.21 ± 9.16	0.573
Vitamin B6 (mg)	1.93 ± 1.34	1.74 ± 0.81	1.84 ± 1.08	0.713
Folate (mcg)	340.02 ± 103.41	250.52 ± 95.79	295.27 ± 107.33	0.060*
B12 (mcg)	4.61 ± 2.83	4.05 ± 2.41	4.33 ± 2.58	0.642
B12, added (mcg)	1.25 ± 2.43	1.03 ± 2.41	1.14 ± 2.58	0.790
Choline (mg)	261.62 ± 89.50	220.77 ± 80.70	241.19 ± 85.55	0.298
Calcium (mg)	734.12 ± 212.29	717.08 ± 319.12	725.60 ± 263.94	0.890
Iron (mg)	12.014 ± 4.18	9.19 ± 3.58	10.60 ± 4.05	0.122
Magnesium (mg)	277.62 ± 107.21	208.27 ± 66.95	242.94 ± 93.99	0.100
Phosphorus (mg)	1102.78 ± 335.95	984.69 ± 421.37	1043.73 ± 375.81	0.497
Potassium (mg)	2323.44 ± 758.70	1814.77 ± 703.89	2069 ± 758.58	0.536
Sodium (mg)	2830.11 ± 871.24	2529.48 ± 1228.33	2679.80 ± 1047.87	0.536

Zinc (mg)	9.92 ± 3.13	8.13 ± 3.68	9.02 ± 3.45	0.257
Copper (mg)	1.25 ± 0.55	0.95 ± 0.27	1.10 ± 0.45	0.141
Selenium (mg)	85.19 ± 27.66	73.08 ± 36.40	79.14 ± 32.07	0.413
Caffeine (mg)	189.19 ± 111.93	120.59 ± 109.75	155.16 ± 113.57	0.180

Table 12: FFQ: Physical Activity

Physical Activity				
Category (per day)	**Treatment Group (mean ± std.)**	**Placebo group (mean ± std.)**	**Total Cohort (mean ± std.)**	**P***
Estimated energy expenditure, all activities (kcals)	497.85 ± 331.73	939.10 ± 662.21	730.09 ± 565.16	0.089
Estimated energy expenditure, excluding work and chores (kcals)	153.62 ± 155.49	254.38 ± 306.67	206.65 ± 245.84	0.388
Estimated light activity (minutes)	78.36 ± 60.50	101.23 ± 73.23	89.79 ± 66.42	0.456
Estimated moderate activity (minutes)	36.09 ± 31.62	56.93 ± 92.08	46.51 ± 67.86	0.507
Estimated vigorous activity (minutes)	11.29 ± 15.25	25.60 ± 28.79	18.44 ± 23.59	0.182
Estimated recreation (minutes)	23.17 ± 19.51	24.61 ± 29.01	23.89 ± 24.07	0.898

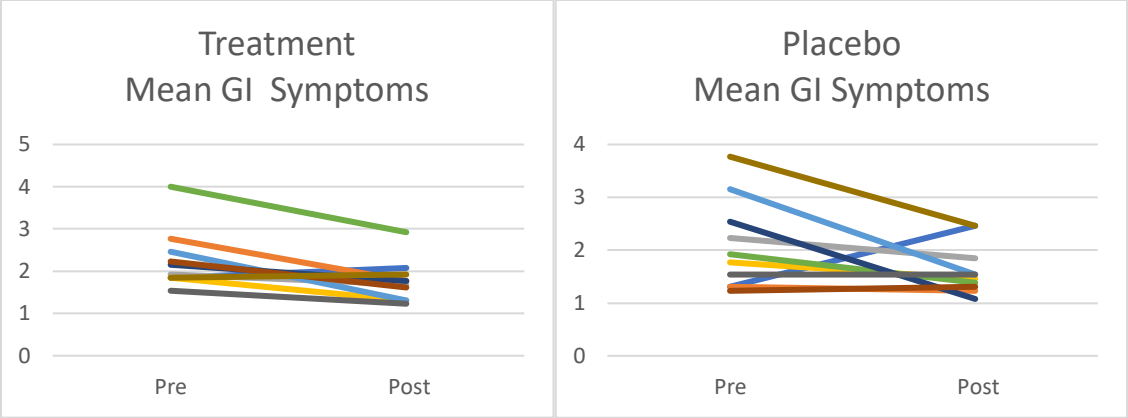
Gastrointestinal Symptoms

Participants completed a gastrointestinal symptoms (GIS) questionnaire using a 7-point Likert scale. Symptoms measured included heartburn, acid regurgitation, bloating, nausea, abdominal distension, eructation, increased gas, decreased passage of stools, increased passage of stools, loose stools, hard stools, urgent need for defecation, and feeling of incomplete evacuation. Out of a possible 91 points, the participants averaged a GIS score of 28.9 ± 9.65. The treatment group had a pre mean GIS score of 29.4 ± 9.18 and a post mean GIS score of 22.9 ±

6.42 (p=0.083091). The placebo group had a pre mean GIS score of 28.4 ± 10.57 and a mean post GIS score of 21.2 ± 6.29 (p=0.080676). A mean reduction of 6.5 points was observed in the treatment group, while a mean reduction of 7.2 points was observed in the placebo group. A significant reduction in bloating (3.6 ± 1.65 vs. 2.3 ± 0.82, p=0.038475) was observed in the treatment group. An approaching significant reduction was observed for loose stools in the treatment group (2.5 ± 1.18 vs. 1.6 ± 0.84, p=0.065169). An approaching significant reduction was also observed in the placebo group for bloating (3.1 ± 1.66 vs. 1.9 ± 0.99, p=0.065897) and increased gas (2.1 ± 1.45 vs. 1.8 ± 1.30, p=0.066324). Starting values from both groups were compared and no significant differences were observed for heartburn (p=0.47), acid regurgitation (p=0.18), bloating (p=0.51), nausea (p=0.38), abdominal distension (p=0.62), eructation (p=0.31), increased gas (p=0.89), decreased passage of stools (p=0.89), increased passage of stools (p=0.52), loose stools (p=0.60), hard stools (p=0.70), urgent need for defecation (p=0.79), feeling of incomplete evacuation (p=0.64), or total GIS (p=0.82). Change from pre to post and significance for the treatment and placebo groups are shown in Table 13.

Table 13: Gastrointestinal Symptoms

Gastrointestinal Symptoms							
Symptom	**Treatment Pre (mean ± stdev.)**	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
Heartburn	1.6 ± 0.97	1.1 ± 0.32	0.137	2.0 ± 1.41	1.8 ± 0.79	0.701	0.569
Acid Regurgitation	1.1 ± 0.32	1.2 ± 0.63	0.660	1.5 ± 0.85	1.6 ± 1.07	0.820	1.000
Bloating	3.6 ± 1.65	2.3 ± 0.82	0.038*	3.1 ± 1.66	1.9 ± 0.99	0.066*	0.894
Nausea	1.4 ± 0.96	1.2 ± 0.42	0.556	1.8 ± 1.03	1.1 ± 0.32	0.055*	0.216
Abdominal Distension	2.9 ± 1.45	1.9 ± 1.29	0.120	2.5 ± 2.01	1.4 ± 0.52	0.112	0.889
Eructation	2.9 ± 1.91	2.0 ± 1.33	0.238	2.1 ± 1.45	1.8 ± 1.03	0.600	0.472
Increased Gas	3.0 ± 1.76	2.2 ± 1.03	0.232	2.9 ± 1.29	1.8 ± 1.30	0.066*	0.675
Decreased Passage of Stools	2.0 ± 1.15	1.9 ± 1.85	0.886	1.9 ± 2.02	1.9 ± 1.60	1.000	0.911
Increased Passage of Stools	1.9 ± 1.10	2.1 ± 1.45	0.732	1.5 ± 1.58	1.5 ± 0.85	1.00	0.812
Loose Stools	2.5 ± 1.18	1.6 ± 0.84	0.065*	2.9 ± 2.02	1.8 ± 1.32	0.167	0.748
Hard Stools	2.0 ± 1.25	1.8 ± 1.23	0.722	1.8 ± 1.03	1.6 ± 0.70	0.618	1.000
Urgent Need for Defecation	2.1 ± 1.60	1.5 ± 0.53	0.274	2.3 ± 1.64	2.1 ± 1.45	0.343	1.000
Feeling of Incomplete Evacuation	2.4 ± 1.35	2.1 ± 1.52	0.647	1.7 ± 1.06	1.3 ± 0.48	0.115	0.247



*Each color in the graph represents an individual participant in the study

Figure 2: Change in Mean GI Symptoms in Treatment and Placebo Groups

The change in treatment group compared to the change in placebo group was insignificant for heartburn (p=0.569275418), acid regurgitation (p=1.00), bloating (p=0.894446423), nausea and vomiting (p= 0.216268549), abdominal distension (p=0.888985282), eructation (0.426753529), increased gas (p=0.674699956), decreased gas (p=0.911541284), increased passage of stools (p=0.811708962), loose stools (p=0.747918259), hard stools (p=1.00), urgent need for defecation (p=1.00), feeling of incomplete evacuation (p=0.246784597), and total GI symptoms (p0.836516559).

POMS Assessment

The Profile of Mood States self-assessment measured initial and change in mood states for anger/ hostility, confusion/ bewilderment, depression/ dejection, fatigue/ intertia, tension/ anxiety, vigor/ activity, and friendliness. Measurements are reported as a t-score calculated by MHS Assessments. The starting mean t-score for both the treatment and placebo groups within each category fell within the typical score range (t-score of 40-59). Additionally, there was no significant change over time in either group, nor was there a significant difference between groups. Change from pre to post and significance for the treatment and placebo groups are shown in Table 14.

Table 14: POMS T-Scores

POMS (T-Score)							
	Treatment Pre (mean ± stdev.)	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
Total Mood Disturbance	44.5 ± 5.44	45.3 ± 9.87	0.825	44.1 ± 4.23	42.2 ± 5.51	0.399	0.373
Anger-Hostility	40.3 ± 2.63	42.5 ± 7.17	0.374	42.1 ± 5.55	40.0 ± 1.76	0.269	0.165
Confusion-Bewilderment	43.2 ± 3.77	43.4 ± 6.15	0.931	42.0 ± 3.94	41.5 ± 3.81	0.776	0.765
Depression-Dejection	43.0 ± 2.0	44.8 ± 7.89	0.493	43.8 ± 3.05	44.0 ± 2.0	0.864	0.508
Fatigue-Inertia	44.4 ± 9.23	44.8 ±10.25	0.928	38.8 ± 5.14	38.3 ± 5.29	0.833	0.712
Tension-Anxiety	41.5 ± 5.40	42.0 ± 7.20	0.862	41.9 ± 4.82	41.9 ± 4.53	1.000	0.864
Vigor-Activity	47.0 ± 7.63	48.8 ±10.80	0.664	44.2 ± 8.35	49.4 ± 8.67	0.189	0.283
Friendliness	53.1 ± 3.48	54.7 ± 9.24	0.614	48.0 ± 8.06	47.6 ± 10.95	0.927	0.549

Blood Analysis

Immunoglobins

The concentration of immunoglobins (ug/mL) IgG1, IgG2, IgG3, IgG4, IgA, and IgM was measured via ELISA analysis of the pre and post blood samples of each participant in duplicate. An approaching significant reduction was observed for IgG1 in the treatment group (7344.04 ± 2381.53 vs. 5094.64 vs ± 2729.10, p=0.0651850) with no significant change from pre to post measured in the placebo group (8986.89 ± 2753.77 vs. 7901.47 ± 2919.83, p=0.429088). A significant reduction was observed in the treatment group for IgG2 from pre to post (7110.44 ± 2437.07 vs. 4185.56 ± 1901.13, p=0.007813) with no significant change observed in the placebo group from pre to post (7748.76 ± 3024.44 vs. 5710 ± 1975.02, p=0.109788). No changes were seen from pre to post in either the treatment or placebo groups for IgG3, IgG4,

IgA, or IgM. No significant changes were observed when comparing the change in the treatment group to the change in the placebo group for all immunoglobins ($p>0.05$). Change from pre to post and significance for the treatment and placebo groups are shown in Table 15.

Table 15: Immunoglobins

Immunoglobins (ug/mL)							
	Treatment Pre (mean ± stdev.)	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
IgG1	7344.04 ± 2381.53	5094.64 ± 2729.10	0.065*	8986.89 ± 2753.77	7901.47 ± 2919.83	0.429	0.304
IgG2	7110.44 ± 2437.07	4185.56 ± 1901.13	0.008*	7748.76 ± 3024.44	5710 ± 1975.02	0.110	0.462
IgG3	693.4 ± 518.92	505.76 ± 451.30	0.400	664.4 ± 362.62	589.29 ± 336.46	0.655	0.443
IgG4	342.4 ± 271.51	257.6 ± 241.49	0.470	585.38 ± 532.20	564.89 ± 533.61	0.936	0.276
IgA	2046.24 ± 711.41	1662.92 ± 835.30	0.284	2316.09 ± 1058.41	2197.42 ± 987.08	0.809	0.301
IgM	2247.4 ± 1458.65	1683.84 ± 1614.82	0.424	2136.4 ± 1373.22	1855.02 ± 976.04	0.623	0.523

Cytokines

The concentration of cytokines (pg/mL) IL-2, IL-6, IL-8, and TNF-a was measured via ELISA analysis of the pre and post blood samples of each participant in duplicate. No significant changes were seen from pre to post in the treatment group for all measures. A significant decrease was measured from pre to post in the placebo group for TNF-a (35.30 ± 8.32 vs. 26.01 ± 7.70, $p=0.018498$). No significant differences were observed when comparing the change in the treatment group to the change in the placebo group ($p>0.05$). Change from pre to post and significance for the treatment and placebo groups are shown in Table 16.

Table 16: Cytokines

Cytokines (pg/mL)							
	Treatment Pre (mean ± stdev.)	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
IL-2	3.07 ± 1.43	2.36 ± 0.64	0.172	2.32 ± 0.82	2.46 ± 0.67	0.663	0.144
IL-6	2.23 ± 0.30	2.21 ± 0.40	0.921	2.21 ± 0.29	2.14 ± 0.37	0.659	0.768
IL-8	10.32 ± 2.78	9.27 ± 2.03	0.344	9.35 ± 1.79	9.35 ± 1.10	0.998	0.288
TNF-a	49.8 ± 39.77	31.70±13.72	0.190	35.30 ±8.32	26.01±7.70	0.018*	0.397

Fecal Analysis

Freeze Drying

No significant change was observed in the dry weight (grams) of the stool sample from pre to post in the treatment (13.19 ± 10.37 vs. 11.45 ± 9.81, p=0.704937) or placebo groups (21.12 ± 15.57 vs. 20.98 ± 15.06, p=0.563617). The treatment group began with a mean percent dry weight of 25.94 ± 5.52 and ended with a mean percent dry weight of 26.87 ± 5.64 (p=0.713393). The placebo group began with a mean percent dry weight of 27.75 ± 5.80 and ended with a mean percent dry weight of 26.04 ± 7.16 (p=0.563617). The delta (change in) dry weight mass was not significant between groups (p=0.756729), nor was the delta % dry weight (p=0.385334). Change from pre to post and significance for the treatment and placebo groups are shown in Table 17.

Table 17: Freeze-Drying Dry Weights

Dry Weight							
	Treatment Pre (mean ± stdev.)	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
Dry Weight (g)	13.19±10.37	11.45 ± 9.81	0.705	21.12 ± 15.57	20.98±15.06	0.984	0.757
Dry Weight (%)	25.94 ± 5.52	26.87 ± 5.64	0.713	27.75 ± 5.80	26.04 ± 7.16	0.564	0.385

Protein

No significant change was observed in the total percentage of protein of the stool sample from pre to post in the treatment (28.32 ± 6.73 vs. 33.39 ± 10.10, p=0.236885) or placebo groups (30.45 ± 5.49 vs. 30.14 ± 8.02, p=0.925755). The change in protein percentage in the treatment group compared to the change in the protein percentage in the placebo group was also non-significant (p=0.28021). Change from pre to post and significance for the treatment and placebo groups are shown in Table 18.

Table 18: Percent Protein

Protein (%)						
Treatment Pre (mean ± stdev.)	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
28.32 ± 6.73	33.39 ± 10.10	0.237	30.45 ± 5.49	30.14 ± 8.02	0.926	0.280

Fiber

Percentage of insoluble, soluble, and total dietary fiber was measured from freeze-dried stool samples using an ANKOM Dietary Fiber Analyzer in duplicate. In the treatment group, no significant change was measured for percentage of insoluble dietary fiber (41.54 ± 10.72 vs 39.92 ± 7.12, p=0.686656), soluble dietary fiber (10.33 ± 4.86 vs. 12.24 ± 6.59, p=0.50162), or total dietary fiber (51.88 ± 10.39 vs. 53.04 ± 7.79, p=0.779015). In the placebo group, there was no significant change in the percentage of soluble dietary fiber (15.49 ± 16.46 vs. 10.51 ± 3.81, p=0.389839) or total dietary fiber (50.37 ± 15.61 vs. 54.43 ± 8.23, p=0.499139); however, there was a significant increase in the percentage of insoluble dietary fiber (34.42 ± 10.67 vs. 43.79 ± 5.48, p=0.032455) in the placebo group. The change in percentage of dietary fiber between groups was significant for insoluble dietary fiber (p=0.011882187), but was insignificant for soluble dietary fiber (p=0.306893128) and total dietary fiber (p=0.68386199). Change from pre to post and significance for the treatment and placebo groups are shown in Table 19.

Table 19: Percent Dietary Fiber

Dietary Fiber (%)							
	Treat-ment Pre (mean ± stdev.)	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
Insoluble (%)	41.54± 10.72	39.92 ± 7.12	0.687	34.42 ± 10.67	43.79 ± 5.48	0.032*	0.012*
Soluble (%)	10.33± 4.86	12.24 ± 6.59	0.502	15.49 ± 16.46	10.51 ± 3.81	0.390	0.307
Total (%)	51.88± 10.39	53.04 ± 7.79	0.779	50.37 ± 15.61	54.43 ± 8.23	0.499	0.684

SCFA

Both concentration (mmol/L) and area percent under the curve were measured for SCFA composition of the freeze-dried stool samples in duplicate. No significant change in area percent under the curve or concentration was measured from pre to post in the treatment group. No significant change in area percent under the curve was seen for the placebo group from pre to post; however, a significant change was observed in the placebo group for concentration of Isobutyrate (0.51 ± 0.13 vs. 0.40 ± 0.14, p=0.078227) and Isovalerate (0.65 ± 0.16 vs. 0.50 ± 0.16, p=0.049512). The change in the treatment group compared to the change in the placebo group approached significance for butyrate concentration (p=0.054600918). Change from pre to post and significance for the treatment and placebo groups are shown in Table 20 and 21.

Table 20: SCFA: Area Percent Under the Curve

Area Percent Under the Curve							
SCFA	**Treatment Pre (mean ± std.)**	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
Acetate	34.66±8.33	32.80 ± 7.65	0.610	32.42 ±5.17	33.26 ±5.03	0.716	0.283
Propionate	21.50±4.07	20.89 ± 3.88	0.734	24.68 ± 4.62	24.96 ± 3.62	0.883	0.525
Isobutyrate	4.16±1.77	4.10 ± 1.79	0.941	4.15 ± 1.44	3.92 ± 1.27	0.708	0.744
Butyrate	25.37±5.88	28.14 ± 5.06	0.274	25.18 ± 8.11	24.41 ± 4.15	0.792	0.174
Isovalerate	7.78 ± 3.95	7.52 ± 3.76	0.882	7.57 ± 3.19	7.06 ± 2.70	0.704	0.811
Valerate	5.09 ± 2.55	4.92 ± 2.18	0.875	5.33 ± 1.13	5.20 ± 1.23	0.788	0.945
Isocaproate	0.19 ± 0.13	0.26 ± 0.23	0.351	0.22 ± 0.19	0.30 ± 0.32	0.462	0.954
Caproate	1.09 ± 1.13	1.18 ± 0.95	0.852	0.42 ± 0.60	0.86 ± 0.82	0.194	0.327
Heptanoate	0.17 ± 0.26	0.06 ± 0.10	0.236	0.02 ± 0.07	0.02 ± 0.07	0.997	0.234
C2+C3+C4	81.53±8.29	81.83 ±8.43	0.937	82.29 ± 5.51	82.64 ± 5.19	0.886	0.979

Table 21: Dietary Fiber: Concentration

Concentration (mmol/L)							
SCFA	**Treatment Pre (mean ± stdev.)**	**Treatment Post (mean ± stdev.)**	**Treat-ment p***	**Placebo Pre (mean ± stdev.)**	**Placebo Post (mean ± stdev.)**	**Placebo p***	**Delta between groups p***
Acetate	13.52 ±7.20	13.89 ± 7.15	0.907	13.04 ± 7.07	10.09± 4.27	0.273	0.159
Propionate	4.39 ± 2.30	4.51 ± 2.29	0.913	4.85 ± 2.37	3.69 ± 1.31	0.192	0.170
Isobutyrate	0.53 ± 0.30	0.57 ± 0.30	0.808	0.51 ± 0.13	0.40 ± 0.14	0.078*	0.124
Butyrate	3.89 ±2.29	4.60 ± 2.70	0.533	3.62 ± 2.28	2.70 ± 1.31	0.282	0.055*
Isovalerate	0.70 ± 0.46	0.74 ± 0.44	0.838	0.65 ± 0.16	0.50 ± 0.16	0.0495*	0.135
Valerate	0.68 ± 0.45	0.69 ± 0.38	0.960	0.67 ± 0.19	0.55 ± 0.17	0.158	0.221
Isocaproate	0.36 ± 0.01	0.42 ± 0.16	0.298	0.33 ± 0.12	0.31 ± 0.12	0.750	0.173
Caproate	0.61 ± 0.21	0.61 ± 0.14	0.958	0.50 ± 0.20	0.52 ± 0.20	0.800	0.856
Heptanoate	0.68 ± 0.62	0.35 ± 0.48	0.188	0.12 ± 0.39	0.12 ± 0.39	0.997	0.259
C2+C3+C4	21.80±10.78	21.07±10.83	0.882	21.51±11.06	16.47±6.59	0.232	0.252
Total SCFA	25.37±11.33	26.38±11.89	0.848	24.29±11.06	16.47±6.59	0.224	0.133

CHAPTER 5

DISCUSSION

Participants

The total sample size for this study was 20, 10 of which were randomized to receive the treatment and 10 of which who were randomized to complete the placebo. A relatively small sample size with a large number of participant variables, such as differences in age, weight classification, lifestyle, and dietary habits could account for small changes in primary outcome measures over time.

Demographics

There were no significant differences for age, gender, or ethnicity between the treatment and placebo groups. That being said, the was a wide standard deviation in the age of the participants in both groups (treatment: 46.80 ± 17.25, placebo: 37.2 ± 16.42). The total cohort had a mean age of 42 ± 17.11. Typically, the prevalence of GI symptoms is higher in adults over age 50. That being said, several of the participants were recruited from East Tennessee State University which may have contributed to a younger participant cohort. Additionally, the majority of participants from each group (70%) were female. This supports research that shows that the prevalence of constipation and diarrhea is higher in women compared to men. Approaching significant differences were seen when looking at the BMI weight classification of the treatment and placebo groups. The treatment group had more normal weight (n=6), an equal number of overweight (n=3), and a lower number of obese (n=1) participants compared to the placebo group (normal weight: n=2, overweight: n=3, and obese: n=5). Since weight classification may influence gut fermentation and the gut microbiome, differences in weight between groups may affect the results of the study.

Assessments

Food Frequency Questionnaire and Physical Activity

Mean daily caloric intake for the total cohort averaged 1543 ± 517.95 calories which is typical for most Americans, particularly because the cohort was comprised of 70% females (n=14) which typically have lower energy requirements compared to males. Interestingly the total cohort had a higher percentage of calories from fat (39.46%) compared to the Dietary Guidelines for Americans recommendation of 20-35% of total calories per day.[75] Calories from saturated fat averaged 193.86 (12.56%) which is slightly higher than the recommended intake of saturated fat (<10%).[75] Additionally, fiber intake for total cohort averaged 15.22 ± 7.45 grams per day which is below the recommended intake of 20-35 grams per day (varies depending on age and sex).[75] Dietary intakes higher in total and saturated fat and lower in fiber compared to the recommended intake can be a contributing factor to gastrointestinal symptoms in this population.

Of importance, there was not a significant difference in dietary intake or composition between groups at the start of the study. Since diet composition influences gut fermentation and the gut microbiome, differences between groups could have skewed the interpretation of results.

Gastrointestinal Symptoms

Out of a possible 91 points, participants in both groups averaged a GIS score of 28.9 ± 9.65. This indicates that participants had relatively mild gastrointestinal symptoms, but each participant met the inclusion criteria of the study in that each person self-reported the presence of at least one GIS three or more times per week. Additionally, there was no significant difference in starting values between groups which allowed for accurate analysis of ending measures of gastrointestinal upset.

A decrease in the presence of total GI symptoms was observed in the treatment group from start to finish and a significant decrease was observed for bloating and incidence of loose stools in the treatment group. These findings support that combination probiotic, prebiotic, and enzyme can improve certain self-reported measures of GIS incidence.

POMS Assessment

Research shows that individuals with GI upset have an impaired quality of life compared to individuals without GI upset. The mean results of the POMs questionnaire for this study; however, suggests that the starting mood state of all participants (n=20) falls within the normal or typical response range. Additionally, a significant change was not observed for any mood state over time in the treatment or placebo group. Future research may consider utilizing the POMs questionnaire as a method of inclusion. For example, 'potential research subjects must exhibit an elevated or very elevated t-score for total mood disturbance to be included'. This would be a better method to evaluate change in mood state as a result of synbiotic supplementation. Ultimately, results of this study suggest that individuals with GI upset do not have an altered mood state; however, the sample size was very small and a larger research study should be conducted to confirm this.

Blood Analysis

Immunoglobins

A significant reduction in serum IgG levels from start to finish in the treatment group could be a serum response to a change in the gut microbiome; however, research is inclusive on whether or not the gut microbiota can provoke systemic change to IgG levels.[76]

Cytokines

One potential effect of probiotic intake is a reduction in serum inflammatory markers. The results of this study do not indicate that GlutenShield has an effect on IL-2, IL-6, IL-8, or TNF-a concentrations. An anomaly was observed in the placebo group, which experienced a significant reduction in TNF-a from start to finish.

Fecal Analysis

Fiber

There are two primary classifications for fiber: insoluble and soluble. Soluble fiber delays gastric emptying and increases transit time (slows movement through GI tract). Insoluble fiber decreases transit time (speeds up movement through GI tract) and increase fecal bulk. Typically, soluble fiber assists with diarrhea while insoluble fiber assists with constipation. Because a common claim associated with consuming probiotics is better bowel regularity, we predicted a change in fecal dietary fiber concentrations as a result of GlutenShield supplementation. That being said, no significant change was observed over time. Fecal dietary fiber concentrations are strongly influenced by one's diet starting and ending values alone are not a good indicator of typical fecal fiber concentrations of those with gastrointestinal upset. The change over time, however, would be a good measurement of effect.

SCFA

One of the primary hypotheses of this research is that synbiotic (prebiotic and probiotic) supplementation would increase fecal SCFA concentrations as an indication of change in total gut fermentation. No significant change was seen in the treatment group for any SCFA over time and there was no significant change between groups for SCFAs. These results indicate that there was no change in fermentation from start to finish. While probiotics may have an indirect effect

on SCFA concentration, prebiotics, in particular, have been shown to have the greatest potential to increase SCFA production and as a result, affect the microbial composition of the gut. Different prebiotic fibers have different effects on SCFA production. That being said, the GlutenShield supplement contained several different types of prebiotics (Chitosan oligosaccharide, fructooligosaccharides, alfalfa, Emblica officinalis extract, papaya juice powder, fulvic acid, and ionic minerals). The GlutenShield supplement may need increased amounts of prebiotics or a longer period of supplementation for changes to SCFA concentrations to be observed.

CHAPTER 6

CONCLUSION

Research findings suggest that GlutenShield was well tolerated and perceived to be beneficial compared to the placebo. Analysis of the dietary recall shows that the participants had higher consumption of total fat and saturated fat while they had lower consumption of dietary fiber compared to the recommendations for Americans. Additionally, intake of grains, whole grains, fruit, dairy, and beneficial oils fell below the recommended intake. These dietary patterns may be a contributing cause of gastrointestinal upset in the population. Results suggest that individuals with minor GI upset do not have altered mood states. Additionally, the mood state is not affected by GS supplementation. Blood analysis showed unremarkable changes to inflammatory markers; however, a significant reduction in IgG levels was observed in the treatment group. This reduction could be in response to a change in the gut microbiome; however, no definitive statements can be made about this change. Last, no changes to gut fermentation (SCFAs) were observed following GS supplementation.

There were several limitations to this study. First, the sample size of the study was fairly small (n=20). While this is large enough to achieve statistical significance, a larger study may be needed to observe true effects of GS supplementation. Second, while it takes a minimum of two weeks to observe changes to fermentation and the gut microbiome, increasing the length of study to greater than a month may result in a larger improvement in measurements of change. Third, demographically, there was a wide range of variability in age, gender, and weight classification which can all impact the results of the study. And fourth, while we had planned to include microbiome analysis in this study, time constraints of thesis completion resulted in an inability to

complete the analysis. Microbiome analysis is currently being completed; however, data is not available to make a definitive conclusion on the effects of GlutenShield on the gut microbiome.

Further research is undeniably needed in the area of prebiotic, probiotic, and enzyme supplementation with consideration to the effect on the human gut microbiome, particularly in those with gastrointestinal upset. Chronic gastrointestinal upset effects ~33.8% of the population and results in significant number of healthcare visits and hospitalizations annually and synbiotics may be a novel therapy for GI upset. Additionally, supplemental use of probiotics, prebiotics, and enzymes is growing and practitioners must have evidenced-based research to support their use in a clinical setting. Larger studies with less demographic variables along with future analysis of microbiome data could provide additional insight into the effects of probiotic, prebiotic, and enzyme supplementation in individuals with gastrointestinal upset. Additionally, studies targeting specific gastrointestinal symptoms (only constipation or only diarrhea) or gastrointestinal conditions (i.e. gluten sensitivity) may result in more conclusive data.

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APPENDICES

Appendix A

Flyer

Version 2 1/3/18

COLLEGE of CLINICAL and REHABILITATIVE HEALTH SCIENCES
EAST TENNESSEE STATE UNIVERSITY

GASTROINTESTINAL SYMPTOMS
Clinical Research Study

Research study conducted out of ETSU College of Clinical and Rehabilitative Health Sciences in conjunction with the College of Health Sciences.

Looking for individuals with gastrointestinal upset who are interested in testing a new product.

Interested individuals must exhibit gastrointestinal upset such as:

- **Bloating**
- **Diarrhea (loose stool)**
- **Constipation (hard stool)**
- **Increased gas**
- **Abdominal discomfort/ pain**
- **Nausea/ vomiting**

Interested individuals cannot have:

- **Celiac disease**
- **Inflammatory bowel disease**
  - **Crohn's disease**
  - **Ulcerative colitis**
- **Short bowel syndrome**

Participants must be 18 years or older.

All participants will receive information about their gastrointestinal function and will receive a 30-day supply of the product after the trial.

If you are interested in evaluating a new product that may provide further understanding of the role of prebiotics and probiotics, please contact:

Kaitlyn Webb
Dietetic Intern/ Graduate Student in Clinical Nutrition
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ETSU

Kaitlyn Webb (423) 946-0977 webbkm1@etsu.edu

Appendix B

Phone Interview

Phone Interview

Name: Phone: Email:

Preferred method of administering results:

Phone Email In person

Inclusion		
Are GI symptoms present more than 3 times per week (i.e. abdominal pain, heartburn, acid regurgitation, bloating, nausea and vomiting, abdominal distension, burping, increased gas, decreased passage of stools, increased passage of stools, loose stools, hard stools, urgent need for defecation, feeling of incomplete evacuation)?	Yes	No
Do you identify as healthy (few health complications)?	Yes	No
Are you over the age of 18?	Yes	No

Exclusion		
Are you under the age of 18?	Yes	No
Do you have Celiac disease?	Yes	No
Do you have IBS?	Yes	No
Do you have IBD? Crohn's or UC?	Yes	No
Have you previously taken GlutenShield?	Yes	No
Are you currently taking prebiotics, probiotics, enzymes, NSAIDS (e.g. Ibuprofen/ Advil, aspirin, naproxen/ Aleve, Celebrex, Midol), fish oil, or fiber supplements?	Yes	No
Have nonsteroidal anti-inflammatory drugs, such as Aspirin, Ibuprofen, Advil, Aleve, or Midol, been	Yes	No

recommended by a health care provider?		
If yes to the above two questions are you willing to halt use for the 6 weeks of the study? (not applicable if recommended or prescribed by a physician)	Yes	No
Are you pregnant or do you intend to become pregnant within the next 60 days?	Yes	No

- Does the participant meet the inclusion and exclusion criteria above?
  Yes No
- If yes, is the participant willing to meet to complete an informed consent?
  Yes No
- Is the participant willing to drive to ETSU on 2 occasions?
  Yes No
- Is the participant willing to submit 2 stool samples, have 2 samples of blood collected, complete questionnaires, and take GlutenShield/placebo for 28-days?
  Yes No
- Is the participant available for the foreseen duration of the study (total of 6 weeks after all participants are recruited)?
  Yes No

By signing below, you are agreeing that the information collected during the phone interview is correct.

_____ _____
Signature of Participant Date

_____ _____
Printed Name of Participant Date

_____ _____
Signature of Principal Investigator Date

Appendix C

Stool Collection Procedure Form

Stool Collection Procedure Form

Materials Provided:

- Saran (plastic) wrap
- Self-sealing plastic bag
- Gloves

Procedure:

1. Lift the toilet seat and put a layer of saran (plastic) wrap across the toilet, leaving a small dip in the middle.
2. Deposit the stool sample onto the saran wrap.
3. Lift the toilet seat and wrap the stool sample in the saran wrap.
4. Place the stool sample in a self-sealing plastic bag.
5. Store the sample in the freezer and bring the sample with you when you come to ETSU on days 0 and 28 of the active phase of the study.

*Note: please attempt to collect the stool sample within 24-hours of submitting the sample.

VITA

KAITYLYN WEBB

Education:	M.S. Clinical Nutrition, East Tennessee State University, Johnson City, Tennessee, 2019 B.S. Food and Nutrition, East Tennessee State University, Johnson City, Tennessee 2017
Professional Experience:	Dietetic Trainee, Mountain Home Veteran Affair Healthcare System, 2018-2019 Graduate Assistant, East Tennessee State University, College of Clinical and Rehabilitative Sciences, 2017-2018 Graduate Assistant, East Tennessee State University, Center for Academic Achievement, 2017-2018
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