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The Effect Of Short-Term Supplementation Of Potato Starch And Vsl#3 On The Large Intestines Of Male C57bl/6 Mice

Kaitlyn R. Merz
Wayne State University, kaitlynmerz@gmail.com

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THE EFFECT OF SHORT-TERM SUPPLEMENTATION OF POTATO STARCH AND VSL#3 ON THE LARGE INTESTINES OF MALE C57BL/6 MICE

by

KAITLYN R. MERZ

THESIS

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Approved By:

________________________________________  ____________________________
Advisor  Date
ACKNOWLEDGEMENTS

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CHAPTER 1 INTRODUCTION

The gut microbiome is one of the body’s most essential organs, harboring approximately 100 trillion microbes and greater than 100-fold more genes than the human host DNA [1]. This population is constructed of over 1,000 species of bacteria from few phyla [2]. The microbiome serves a wide array of functions involved in digestion [3], metabolism [4], immune system development [5], and cognitive function [6]. Dysbiosis, or microbial imbalance in the gut [7], is found to potentially influence the prevalence of diseases such as obesity [8], inflammatory bowel disease (IBD) [9], irritable bowel syndrome (IBS) [2], cardiovascular disease (CVD) [10], type 2 diabetes [2], and colorectal cancer [11].

Bacteria of the Gut Microbiome:

As previously mentioned, there are trillions of bacteria present in the gastrointestinal (GI) tract. These microbiota have a vast array of functions, most of which are still unknown. Colonization begins immediately following birth and undergoes major changes to the adult microbiome and lastly when the host enters old age [12]. The majority of bacteria in the gut belong to the phyla Bacteroidetes and Firmicutes, and fewer associated with Actinobacteria and Proteobacteria [13].

Enterobacteriaceae is a member of the Proteobacteria phyla present in the GI tract. This bacteria is highest following birth then decreases during transition to adulthood [7]. Enterobacteriaceae in an increased amount has been associated with dysbiosis seen in metabolic and inflammatory disorders [14, 15]. Abundance of Enterobacteriaceae in the digestive tract is possibly a result of a diet higher in calories and fat and lower in dietary fiber [16]. Enterobacteriaceae can be considered a helpful marker of GI microbiome health by observing elevation in certain diets and diseases.
Specific species of gut bacteria are thought to serve several benefits to the host. Two of the most common beneficial gut bacteria, also widely used in probiotic supplements, include *Lactobacillus* and *Bifidobacterium* [2]. Both are gram positive, rod, and lactic acid producing bacteria that have the ability to support digestion and facilitate break down of carbohydrates [17-19]. *Lactobacillus*, belonging to the *Firmicutes* phyla, is predominant in the small intestine and also fairly common in the large intestine and feces [20]. This species has exhibited an ability to regulate the intestinal environment and maintain eubiosis [21]. *Lactobacillus* is commonly found in milk and yogurt and is believed to assist in lactose digestion by serving as a source of lactase in the small intestine [18]. *Bifidobacterium*, from the *Actinobacteria* phyla, is one of the first bacteria to colonize after birth and are present in elevated amounts in breast milk, preventing GI diseases in infants [22]. This species has demonstrated the capability to protect the digestive system by inhibiting growth of pathogens [23]. *Bifidobacterium* is also capable of repair in the large intestine and lessen GI symptoms in those with GI disorders [24]. Both groups of bacteria have shown many benefits among the GI tract. Therefore, there is an importance in investigating the growth and colonization of bacteria inhabiting the gut.

**Large Intestine:**

The large intestine is home to the majority of microbiota containing $10^4$ to $10^{12}$ colony forming units per milliliter, presumably due to slower transit of ingested material [13]. Bacteria residing in the large intestine consists mostly of anaerobes, while facultative anaerobes and aerobes exist in much smaller amounts [2]. It is here where non-digestible dietary components, mainly carbohydrates, are broken down and fermented by bacteria, synthesizing short chain fatty acids (SCFA), organic acids, and alcohols [25]. Decreased diversity of microbiota among the large intestinal microenvironment can lead altered production of fermentation products, inflammation
and ultimately gastrointestinal disease [2]. Therefore, there is great importance in maintaining healthy gut microbiota balance, also known as eubiosis, in the large intestine and other locations along the GI tract [9].

**pH of the Large Intestine:**

Large intestine pH is an indicator of health and current state of the microenvironment [26]. The pH is generally neutral, influenced by fermentation products and effects growth of certain bacteria [27]. A reduced pH allows for inhibition of pathogens, prevention of carcinogenesis, and increased fermentation of non-digestible carbohydrates, promoting beneficial effects, unlike an increased pH, which is linked to development of colon cancer [28]. With a reduction in pH, bacteria present in lower amounts with higher tolerance to pH alterations, such as *Bifidobacterium*, can outgrow the usual dominant groups of bacteria [26, 29]. The pH can contribute to bacterial growth, overall health, and represent health status of the large intestine.

**Microbiota Related Diseases:**

An unhealthy gut has been associated with diseases of the large intestine. Inflammatory bowel disease (IBD) is a common inflammatory disease related to chronic inflammation in the GI tract, mainly in the large intestine [2]. Microbial disturbances along with genetic factors have been associated with the development of IBD, although exact pathogenesis is unknown [30]. There are two forms of IBD consisting of ulcerative colitis, characterized by chronic inflammation of the large intestine, and Crohn’s disease, distinguished by transmural inflammation [31]. IBD has been linked to increased incidence of colorectal cancer possibly by initiating tumorigenesis in the large intestine [11]. Irritable bowel syndrome (IBS) is another disease of the gut that’s pathogenesis is also unknown. IBS is related to low grade inflammation and decreased microbiota diversity portrayed by diarrhea, constipation, or both [2]. Reducing inflammation and maintaining a healthy
balance in microbiota is important in alleviating symptoms and preventing IBD, colorectal cancer, and IBS [9].

**Diet and Microbiota:**

Diet is a critical environmental influencer of GI microbiota contents. Long-term and short-term dietary changes lead to alterations to the microbiome [2]. Diet has the ability to change several aspects of the large intestine microenvironment including pH, bile salt concentration, and micronutrient concentration, all important to the survival and function of microbiota [4]. However, only certain species are sensitive to dietary changes due to variance in tolerance to changes in the microenvironment of the large intestine [4]. For example, the Western diet is high in total fat, refined sugars, and animal protein [32]. Diets possessing these features have been related to dysbiosis, leading to inflammation in the large intestine, potentially causing inflammatory diseases and colorectal cancer [32]. In contrast, diets rich in dietary fiber are associated with less prevalence of inflammatory diseases [16]. Therefore, it can be assumed that the diet has an immense influence on the contents and microenvironment of the gut microbiome.

**Prebiotics:**

A prebiotic is a dietary ingredient fermented in the large intestine that allows for alterations in the microbiota and poses benefits on the host [2]. Resistant starch, considered a prebiotic, acts as a dietary fiber that resists digestion by α-amylase in the small intestine, reaching the large intestine intact, where it is fermented by microbiota and has beneficial effects [25]. Supplementing the diet with resistant starch has shown to increase fecal bulk, reduce transit time, decrease colonic pH, and enhance growth of beneficial microbiota [25]. Raw potato starch is a resistant starch that is easily accessible and inexpensive. Raw potato starch is considered a resistant starch type 2 (RS2), which is protected from digestion because of its crystalline structure [25]. RS2 may
possibly prevent DNA damage found in colon cancer through desmutagenesis [33] as well as increasing growth of beneficial bacteria in the large intestine [34]. These effects allow for better health of the large intestine and decreased risk of diseases.

**Probiotics:**

Probiotics have also shown beneficial effects on the large intestine. Most probiotics contain bacterial strains from *Bifidobacterium* and *Lactobacillus* [2]. The benefits of probiotics are thought to be from the products of the bacteria rather than the bacteria itself [35]. Probiotics have been shown to be beneficial in treating large intestinal diseases, however, it is unclear if they are effective in healthy individuals [3]. The widely studied probiotic mixture VSL#3 was able to successfully alter microbiota composition and reduce inflammation in the large intestine alleviating symptoms of bowel diseases [35]. VSL#3 is a high potency probiotic comprised of three strains of *Bifidobacterium* (*B. breve, B. longum, B. infantis*), four strains of *Lactobacillus* (*L. acidophilus, L. plantarum, L. paracasei, L. delbruekii*), and *Streptococcus thermophiles* [36]. This probiotic is recommended by the American Gastroenterology Association for remission and maintenance of large intestine diseases [37]. VSL#3 could possibly decrease inflammation and enhance epithelial barrier function, which dysfunction has been present in disease [38]. VSL#3 supplementation has also assisted in relieving functional constipation by increasing complete spontaneous bowel movements in patients [39]. VSL#3 has aided in diseased and could potentially promote microbiota in healthy individuals.

**Inflammatory Cytokines:**

Aside from bacteria, inflammatory cytokines can be biomarkers of large intestinal health. Cytokines control development, growth, activation, and function of immune cells across the entire body [11]. There are two major types of inflammatory cytokines, pro-inflammatory and anti-
inflammatory. Pro-inflammatory cytokines tend to recruit immune cells and elevate level of reactive oxygen species production, in turn creating an inflammatory environment [35]. Anti-inflammatory cytokines regulate immune response and suppress production of pro-inflammatory cytokines [40]. Large intestinal inflammation has the ability to alter intestinal barrier function and certain microbiota concentrations and has been associated with IBD and colorectal tumor development [11]. Probiotics, such as VSL#3, have shown to reduce production of some pro-inflammatory cytokines [35] and elevate production of anti-inflammatory cytokines [30].

A major anti-inflammatory cytokine, believed to be increased by VSL#3 supplementation, is Interleukin 10 (IL-10) [41]. This heightened amount was associated with the presence of Bifidobacteria strains when compared with other bacterial groups present in VSL#3 [41]. IL-10 is immunoregulatory and plays a major role in defense against pathogenic bacteria [7]. Deficiency of IL-10 has been related to development of IBD and undesirable shifts of the gut microbiota [42]. Therefore, upregulation of IL-10 has the potential to reduce inflammation and prevent inflammatory diseases and dysbiosis.

The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) is thought to decrease with supplementation of VSL#3 [35]. TNF-α is produced by macrophages and T cells in the intestinal mucosa and stimulates production of other pro-inflammatory cytokines, while also aiding T cells in resisting apoptosis causing an intensified immune response [40]. TNF-α contributes to chronic inflammation, intestinal damage, and potentially tumorigenesis [11]. Elevation of TNF-α is prevalent in patients with IBD and blocking production can alleviate symptoms and prevent colorectal carcinogenesis [43]. Madsen et al showed that an increase in bacterial load, a potential mechanism of VSL#3, decreased TNF-α production [44]. TNF-α can be used as a biomarker of inflammation risk, while also potentially representing concentration of
other pro-inflammatory cytokines since it is responsible for regulating production [40]. Thus, VSL#3 can possibly regulate production of TNF-α, alleviate symptoms, and prevent chronic inflammation within the large intestine.

**Objective:**

Long-term supplementation of the diet with prebiotics and probiotics have shown many alterations in microbiota, pH, and inflammation leading to benefits. However, there is little evidence that supports benefits of short-term supplementation in healthy individuals. This is important to examine because prebiotics and probiotics could potentially prevent large intestine diseases by promoting diversity in the gut microbiome and may also contribute to a desirable microenvironment leading to enhanced large intestine health. The objective of the current study was to determine effects of short-term supplementation of the prebiotic, potato starch and the probiotic, VSL#3 on large intestine health by examining large intestine tissue and digesta contents, including analysis of microbiota, pH, and inflammatory cytokines. The current study was designed to test the hypothesis that supplementing the diet with VSL#3 poses more beneficial effects on large intestine health than potato starch since VSL#3 involves direct ingestion of beneficial bacteria and evidence has been more established by previous studies examining long term effects in subjects with IBD.
CHAPTER 2 MATERIALS AND METHODS

Animals:

Experimental procedures were approved by the Institutional Animal Care and Use Committee. Mouse large intestine samples were obtained from a previous study by Rivas et al [45]. Briefly, twenty-four male C57BL/6 mice from Jackson Laboratory (Bar Harbor, ME) aged three weeks old were acclimated for two days, split into three groups, then received different dietary interventions lasting two weeks. All groups received food and water ad libitum and intake was recorded regularly. The control group received unmodified chow diet, PicoLab Laboratory Rodent 5L0D (LabDiet, St. Louis, MO). The second group received a chow diet supplemented with 10% potato starch (Bob’s Red Mill, Milwaukie, OR). The third group received the same unmodified chow diet as the control with water being supplemented with one packet (450 billion bacteria) VSL#3 probiotic mixture (Sigma-Tau Healthscience, Gaithersburg, MD) dissolved in 1L of water per day. Animals were sacrificed simultaneously following dietary intervention.

Sample Preparation:

Large intestine samples had been stored at -80°C from the previous study. Samples were thawed at room temperature for approximately five minutes. Large intestines were stretched, pinned and cut longitudinally. Digesta contents were removed from the surface of the tissue and stored at -80°C for further analysis. Large intestine tissue was rinsed with cold, sterile 0.1 M phosphate buffered saline (PBS) to remove remaining digesta contents. Three millimeter by 3 millimeter pieces were cut from the proximal colon for DNA extraction [46]. Approximately 60 milligrams of tissue was minced for analysis by ELISA. All tissue samples were stored at -80°C until further analysis.
**Specific Aim 1:** Establish changes in microbiota concentration of large intestine tissue and digesta content based on dietary intervention.

**DNA Extraction:**

Total genomic DNA was extracted from 3mm by 3mm intestinal samples using QIAmpDNA Mini Kit (QIAGEN Inc., Valencia, CA) following the manufacturer's protocol for tissue samples. DNeasy PowerSoil Kit (QIAGEN Inc., Valencia, CA) was used to extract DNA from intestinal digesta matter samples abiding by the manufacturer’s protocol. Tissue and digesta DNA was quantified using NanoDrop 2000 (Thermo Fisher Scientific Inc., Asheville, NC), diluted to 4ng/µl and stored at -20°C until microbial profiling.

**Microbial Identification and Quantification:**

Microbial identification and quantification of four microbial groups (Table 1) was performed by quantitative PCR (qPCR) using a CFX96 Real-Time System (Bio-Rad, Hercules, CA). Samples were run in triplicate in a 96-well plate, to a final volume of 10µl, containing 4ng of template DNA, 5µl SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA), and 0.5µM microbial group specific primers from previous studies (Table 1) [47-49]. Standard curves were developed using microbial group specific reference strains serially diluted 10-fold and normalized to 16S rRNA gene copy number based on Stoddard et al and genome size [50].

Three digesta and tissue DNA samples from each experimental group were randomly selected to verify amplicon size of group specific primers by endpoint PCR, followed by ethidium bromide stained agarose gel electrophoresis using a 100 base-pair ladder. Reaction volume totaled 25µl containing SYBR green master mix and the same concentration of template DNA, primers and reference strains as qPCR. Parameters for both types of PCR analysis were as follows: denaturation 98°C, annealing 57°C, extension 72°C.
**Specific Aim 2:** Determine differences in the large intestinal microenvironment among experimental groups by examining digesta pH and concentration of inflammatory cytokines.

**Large Intestine pH Measurement:**

A portion of digesta matter extracted from the large intestine was weighed and diluted 1:9 with distilled water to represent the pH of the large intestine [28]. The pH value of each sample was measured in triplicate using an Accumet AB150 pH meter (Fisher Scientific Company LLC, Pittsburgh, PA). Mean pH values of each sample were calculated.

**Measurement of Inflammatory Cytokines by ELISA:**

Large intestine tissue was prepared for ELISA based on Medicherla et al [51]. Briefly, approximately 60 milligrams of large intestine tissue were minced and homogenized in a 10% tissue homogenate with cold phosphate buffered saline (PBS) and 1% Halt protease inhibitor cocktail (Thermo Fisher Scientific Inc., Asheville, NC). Followed by centrifugation at 4,000 rpm for 15 minutes. The supernatant was extracted for further evaluation by ELISA. Total protein content was quantified using NanoDrop Lite and normalized for total protein content (Thermo Fisher Scientific Inc., Asheville, NC). Mouse specific ELISA kits for the inflammatory cytokines TNF-α and IL-10 were purchased from Aviva Systems Biology, Corp. (San Diego, CA) and assays were performed according to manufacturer’s instructions. Optical density (OD) was read at a wavelength of 450 nm using an accuSkan™ FC Filter-Based Microplate Photometer (Fisher Scientific Company LLC, Pittsburgh, PA). Relative OD for each sample was calculated by subtracting the mean blank OD from the mean well OD. A standard curve was developed by plotting the mean relative OD verse concentration of each serially diluted standard provided by the ELISA kits. Concentration of each sample was determined by using linear regression of mean sample relative OD against the standard curve.
Statistical Analysis:

All statistical analyses were performed using SPSS Statistics software (Version 24, IBM Corp., Armonk, NY). Significant differences among groups were evaluated using the nonparametric Kruskal-Wallis test with a significance level of 0.05. Bonferroni correction was used for post-hoc analysis to adjust group means for pairwise comparisons.
CHAPTER 3 RESULTS

Animal Characteristics:

Animal characteristic information was obtained from Rivas et al (Table 2) [45]. Daily food intake and weight gain among groups was not significantly different, however daily water intake was increased in the VSL#3 group. Large intestine length was not different among groups but large intestine weight was significantly increased in the potato starch group.

Microbiota Composition in Response to Supplementation of a Probiotic or Prebiotic:

DNA was extracted from 24 large intestine tissue and digesta samples from male C57BL/6 mice and examined by qPCR for differences in microbiota content of four microbial specific groups. Overall, there was more total bacteria in the digesta matter than colonizing in the large intestine tissue. Also, there were more changes in the digesta DNA compared the large intestine tissue.

Among large intestine tissue DNA, shown in Figure 1, there were limited differences in bacterial groups between the control group and groups supplemented potato starch or VSL#3 when analyzed by qPCR. The intervention groups showed more total bacteria (Figure 1a) and Enterobacteriaceae (Figure 1d) than the control, while the potato starch group contained slightly less Lactobacillus (Figure 1c). The VSL#3 group had a higher concentration of Bifidobacterium in the tissue DNA than the control and potato starch groups. DNA extracted from the digesta, shown in Figure 2, comprised of more total bacteria than the tissue DNA, with the potato starch group having the highest relative quantity and the control group with the least total bacteria present (Figure 2a). The largest significant difference was shown in the vast increase of Bifidobacterium in the potato starch group (Figure 2b). Potato starch and VSL#3 supplementation also showed an increase in Enterobacteriaceae when compared to the control (Figure 2d).
Tissue and digesta DNA extracted from nine samples, three randomly selected from each experimental group, was used for endpoint PCR, followed by ethidium bromide stained agarose gel electrophoresis to verify primer amplicon size. Tissue and digesta DNA samples selected from each group expressed the proper amplicon size when compared to the DNA standard. Total bacteria (Figure 3a) displays an amplicon size of 200bp, *Bifidobacterium* (Figure 3b) shows a 242bp amplicon size, *Lactobacillus* (Figure 3c) reveals an amplicon size of 348bp, and *Enterobacteriaceae* (Figure 3d) expresses a 333bp amplicon size, all with sample DNA matching the reference strain. qPCR results were validated through the presence of the bacterial genes displayed on the agarose gel.

**pH Measurement of Large Intestine:**

Large intestine pH was represented by measuring the pH value of digesta diluted in water from each of the 24 samples. pH values of digesta are summarized in Figure 4. The group supplemented with potato starch showed a reduced pH compared to the control and VSL#3 groups. The group supplemented with VSL#3 did not significantly differ from the control.

**Concentration of Inflammatory Cytokines Through Measurement by ELISA:**

An ELISA of large intestine tissue homogenates was performed to quantify the concentration of anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine TNF-α. Relative quantity of IL-10 in large intestine tissue examined by ELISA is shown in Figure 5. The control group showed the greatest quantity of IL-10. Mice supplemented with VSL#3 showed a significant decrease in the anti-inflammatory cytokine compared to the control, while the group that received potato starch displayed slightly more than VSL#3 but less than the control group but was not considered significantly different.
Results from the ELISA for TNF-α are shown in Figure 6. The data indicates that the group receiving VSL#3 supplementation had a decreased amount of TNF-α when compared to the control and potato starch groups. The potato starch group did not significantly differ from the control.
CHAPTER 4 DISCUSSION

As previously mentioned, maintaining eubiosis of microbiota is beneficial to large intestine health. This study attempted to observe an impact of supplementing the diet with a prebiotic, potato starch, and a probiotic, VSL#3. Significant differences among experimental groups in bacteria concentration, pH and inflammatory cytokine concentration were established.

Animal characteristics (Table 2), from a previous study by Rivas et al showed no significant differences in food intake and weight gain among groups, displaying that the dietary intervention did not result in any appetite or weight changes [45]. However, water intake was increased in the VSL#3 group possibly due to change in water consistency [45]. Large intestine length was not significantly different among groups, although large intestine weight was increased in the group supplemented with potato starch presumably due to the increased fecal bulk [25, 45].

Based on the results of the current study, differences among bacteria colonizing in the tissue are minor when comparing the experimental groups. The group supplemented with VSL#3 displayed an increase in Bifidobacterium concentration in the tissue presumably due to the direct ingestion of the bacteria allowing for better colonization [30]. It can be assumed that supplementing the diet with VSL#3 or potato starch short term does not result in major differences in microbiota residing in the tissue of the large intestine (Figure 1). The large intestine tissue also harbors less total bacteria than the digesta matter.

There were limited differences between the control and VSL#3 groups in digesta bacteria with only minor increases in total bacteria and Enterobacteriaceae with VSL#3 supplementation (Figure 2). It is possible that short-term supplementation of VSL#3 has limited effects on microbiota quantity in healthy mice. Likewise, Kim et al showed no significant changes in
Lactobacillus and Bifidobacterium with short-term supplementation in healthy individuals and those that experienced dysbiosis [39].

The group supplemented with 10% potato starch exhibited a significant increase in total bacteria, Enterobacteriaceae and Bifidobacterium in digesta DNA (Figure 2). The slight elevation in Enterobacteriaceae concentration can be considered abnormal (Figure 2d). De Filippo et al observed differences in microbiota of African children, who consumed more dietary fiber, and less fat, and European children, who consumed high fat, low fiber, and overall more processed, high calorie foods. The results showed that the African children had less Enterobacteriaceae in the gut related to the higher consumption of non-digestible carbohydrates [16]. However, the increase in the potato starch group is minor so it is possible that since supplementation was short-term, the reduction in Enterobacteriaceae had not yet occurred.

The greatest difference examined in bacteria concentration is the high amount of Bifidobacterium in the group supplemented with potato starch (Figure 2b). This is potentially due to the increase in fermentation allowing for stimulation of Bifidobacterium growth, most likely because these strains have a greater capacity to hydrolyze resistant starch [52]. Resistant starch supplementation can cause shifts in microbiota within a few days, which is feasibly why there were more differences observed in the potato starch group [4]. The significant decrease in pH observed in the potato starch group is possibly associated with the increased growth digesta Bifidobacterium (Figure 4). The increase in fermentation can result in a decrease in pH of the large intestine and also make a more desirable growth environment for Bifidobacteria strains and less desirable for Lactobacilli strains due to increase of Bifidobacterium presence [25, 29]. Belenger et al demonstrated that Bifidobacterium became the dominant group with a reduced pH and addition of polysaccharides [26]. Slight alterations in pH, as little as a 0.5-1 unit shift, have shown changes
in the intestinal microenvironment and certain microbiota concentrations, therefore, potato starch supplementation potentially creates a physiologically significant modification in the large intestine pH[29]. With this evidence, it can be presumed that potato starch supplementation is capable of altering microbiota and ultimately pH in the large intestine of healthy mice with short-term supplementation.

Rivas et al examined total bacteria, *Lactobacillus* and *Bifidobacterium* in fresh feces of the same mice by qPCR, using identical group specific primers as the present study [45]. When comparing bacteria in feces to digesta differences can be observed. Rivas et al showed that total bacteria among experimental groups were not significantly different in contrast to the present study, which displayed all groups total bacteria concentration were significantly different. In addition, Rivas et al exhibited that fecal *Lactobacillus* in the VSL#3 group were significantly different compared to other groups, dissimilarly to the present study that presented no significant differences in digesta *Lactobacillus*. *Bifidobacterium* concentration in feces and digesta revealed similar results. Gu et al inspected the differences in bacteria between large intestine contents and feces of C57BL/6 mice, resulting in differences among bacteria concentration of certain groups between the samples [20]. Therefore, it is possible the composition of feces and large intestine digesta are slightly different. The differences could have potentially been a result of continuous exposure to nutrients and possible colonization into tissue of the distal colon as the digesta was transited out and converted to feces [53]. Perhaps feces are not a direct representation of microbiota in the large intestine.

IL-10 is an anti-inflammatory cytokine that has regulatory effects on the immune system and can be altered in response to changes in the microbiota [7]. The outcome of the ELISA performed on large intestine tissue homogenates showed the VSL#3 group had significantly less
than the control group, which contained the highest concentration of IL-10 (Figure 5). Previous studies have displayed induced production of IL-10 in intestinal cells with VSL#3 supplementation [41, 54]. This decrease in IL-10 could potentially explain the elevation in tissue and digesta Enterobacteriaceae in the VSL#3 group since IL-10 deficiency has been associated with increased colonization of Proteobacteria [7]. In contrast, increased Bifidobacterium concentration, also seen in the tissue of the VSL#3 group, has previously resulted in increased IL-10 concentration [30]. Hart et al expressed that an increased presence of Bifidobacterium breve and infantis, found in the VSL#3 supplement have anti-inflammatory effects and can increase concentrations of IL-10 [41]. However, this study was performed in vitro on large intestine tissue biopsies in the presence of VSL#3 strains. It is possible that this is not effective in vivo, or perhaps the slight increase in Bifidobacterium in the VSL#3 group in large intestine tissue (Figure 1b) and an increase in the digesta Bifidobacterium of the potato starch group (Figure 2b) were not the strains found in the VSL#3 supplement, therefore, did not result in an increase of IL-10. Future research on exact strains of Bifidobacteria and concentration needed in vivo will be required.

Studies observing an increase in IL-10 secretion with VSL#3 supplementation are examining subjects with inflammatory diseases resulting from an abnormal immune response. One of the many functions of IL-10 is inhibition of pro-inflammatory cytokines and in opposition, TNF-α is responsible for stimulating production of pro-inflammatory cytokines [40]. There are several pathways that regulate production of TNF-α and IL-10, most of which are related to the level of immune response [40, 55]. It is evident that VSL#3 suppresses immune response by regulating cytokine production and reducing pro-inflammatory cytokines in IBD [35]. It is plausible that VSL#3 has the ability to do the same in subjects with normal immune function based on the reduction of TNF-α in the group supplemented with VSL#3 (Figure 6). With the reduction in TNF-
α it is possible that there is a reduction in other pro-inflammatory cytokines, therefore a reduction in overall immune response [40]. This is a possible reason for the reduction in IL-10 since several of the regulation pathways begin with the immune response and is thought to be induced in the presence of elevated pro-inflammatory cytokines [55].

Other studies examining long-term effects of VSL#3 showed more variation in bacteria concentration and increased IL-10 in IBD, differing from the present study. The results of the present study do not support the original hypothesis with VSL#3 showing minor changes in microbiota concentration and reduced IL-10 when compared to potato starch supplementation. In conclusion, potato starch and VSL#3 have benefits when supplemented short-term, however the effects of each supplementation are different. Potato starch has the ability to increase fermentation in the large intestine, ultimately increasing *Bifidobacterium* in the digesta and decreasing the pH of the large intestine. Short-term VSL#3 supplementation has possible effects of decreasing immune response and reduce the pro-inflammatory cytokine TNF-α. Future analysis could examine these effects long-term in healthy subjects and the possible effects of combining VSL#3 and potato starch symbiotically in one dietary intervention to establish simultaneous or more beneficial effects.
## Table 1: Microbial Group Specific Primers and Genomic Standards for Quantitative and Endpoint PCR

<table>
<thead>
<tr>
<th>Microbial Group</th>
<th>Primer (5’-3’)</th>
<th>Genomic Standard</th>
<th>Amplicon Size(bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus</em></td>
<td>F: GCAGCAGTGGGAATCTTCCA R: GCATTYACCGCTACACATG</td>
<td>Lactobacillus rhamnosus</td>
<td>348</td>
<td>Castillo et al, 2006</td>
</tr>
</tbody>
</table>
Table 2: Animal Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Potato Starch</th>
<th>VSL#3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Daily Food Intake (g)</strong></td>
<td>3.36 ± 0.26</td>
<td>3.70 ± 0.36</td>
<td>3.74 ± 0.29</td>
</tr>
<tr>
<td><strong>Daily Water Intake (mL)</strong></td>
<td>5.54 ± 0.77</td>
<td>5.55 ± 1.21</td>
<td>6.65 ± 1.39*</td>
</tr>
<tr>
<td><strong>Weight Gained (g)</strong></td>
<td>7.71 ± 0.94</td>
<td>8.65 ± 0.71</td>
<td>8.27 ± 1.33</td>
</tr>
<tr>
<td><strong>Large Intestine Weight (g)</strong></td>
<td>0.95 ± 0.08</td>
<td>1.23 ± 0.10*</td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td><strong>Large Intestine Length (cm)</strong></td>
<td>10.10 ± 0.60</td>
<td>10.70 ± 0.90</td>
<td>10.50 ± 1.0</td>
</tr>
</tbody>
</table>

All information on this table was obtained from Rivas et al [45]. Values expressed as per mouse in mean ± standard deviation. Significantly different values are labeled with * based on a significance level of 0.05.
Figure 1: Relative Quantity of Bacterial Groups in Large Intestine Tissue DNA from qPCR. Relative quantity of each bacterial group is provided in log 16S rRNA gene copies per nanogram of total DNA. Significance was determined using a p value less than 0.05, experimental groups containing different letters are considered significantly different.
Figure 2: Relative Quantity of Bacterial Groups in Digesta DNA from qPCR. Relative quantity of each bacterial group is provided in log 16S rRNA gene copies per nanogram of total DNA. Significance was determined using a p value less than 0.05, experimental groups containing different letters are considered significantly different.
Figure 3: Endpoint PCR Ethidium Bromide Stained Gel Electrophoresis. Tissue and fecal DNA are represented on an ethidium bromide stained gel using a 100 base-pair ladder. Three samples randomly selected from each experimental group are labeled T (tissue DNA), D (digesta DNA), with C (control), P (potato starch), and V (VSL#3). NTC represents the no template control and the reference strain is labeled accordingly. Amplicon sizes are as follows: Total bacteria (a) 200bp, Bifidobacterium (b) 242bp, Lactobacillus (c) 348bp, and Enterobacteriaceae (d) 333bp. All with sample DNA matching the reference strain genome size.
**Figure 4: Digesta pH Values.** pH values of each experimental group are represented by the mean pH of three replicates in each group. Significance was determined with a p value less than 0.05, groups containing different letters are significantly different.
Figure 5: Relative Quantity of IL-10 in Large Intestine Tissue. Mean quantity of three replicates of each sample among three experimental group of IL-10 is expressed in picograms per milligram of protein relative to the standard curve values. Significance was determined using a p value less than 0.05, groups containing different letters are significantly different.
**Figure 6: Relative Quantity of TNF-α in Large Intestine Tissue.** Mean quantity of three replicates of each sample among three experimental group of TNF-α is expressed in picograms per milligram of protein relative to the standard curve values. Significance was determined using a p value less than 0.05, groups containing different letters are significantly different.
REFERENCES


ABSTRACT

THE EFFECT OF SHORT-TERM SUPPLEMENTATION OF POTATO STARCH AND VSL#3 ON THE LARGE INTESTINES OF MALE C57BL/6 MICE

by

KAITLYN R. MERZ

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Advisor: Dr. Yifan Zhang

Major: Nutrition & Food Science

Degree: Master of Science

The microbiome is extremely complex and presumed to be involved in several biological processes. Dysbiosis is associated with development of several diseases, therefore, eubiosis is essential for prevention and treatment, possibly achieved through prebiotic or probiotic supplementation. The objective of this study was to establish effects of short-term supplementation on large intestine microbiota, pH and inflammatory cytokines. Large intestines of 21 day old C57BL/6 male mice that were given a control diet or supplemented with 10% potato starch or VSL#3 short-term, were analyzed for shifts in bacteria, pH, and inflammatory cytokine concentration. Large intestine digesta bacteria concentration differed from feces from previous study results, possibly due to colonization and exposure to nutrients, potentially meaning feces is not a direct representation of large intestine microbiota concentration. In conclusion, both supplementations exhibited potential mechanisms to promote large intestine health. Short-term potato starch supplementation caused an increase in beneficial Bifidobacterium in digesta, potentially due to increased fermentation, leading to a decrease in pH. Short-term VSL#3 supplementation displayed a reduction in both cytokines IL-10 and TNF-α in large intestine tissue, suggesting a decreased immune response. This is possibly due to the decreased TNF-α causing a
reduction in all pro-inflammatory cytokines, ultimately leading to less need of anti-inflammatory IL-10, which suppresses pro-inflammatory cytokine production. In conclusion, both supplementations displayed benefits short-term, however further analysis is needed potentially examining long-term supplementation, in IBD subjects, or symbiotically.
AUTOBIOGRAPHICAL STATEMENT

Kaitlyn R. Merz

Education:

Wayne State University

Master of Science 2016-2017
Nutrition & Food Science Detroit, MI

Western Michigan University

Bachelor of Science 2012-2016
Dietetics Kalamazoo, MI