Lauren Kitts

Introduction

Inflammatory bowel disease (IBD) is a widespread disease that affects 3.1 million people in the United States alone (CDC, 2019). IBD is a chronic immune mediated condition with relapsing colitis, or inflammation, which affects the intestinal mucous lining (Carding, 2015). The symptoms range from abdominal pain to bowel obstructions, and in rare cases, even death (Feuerstein, 2017). Inflammatory bowel disease has put a major strain on the global healthcare system and accounts for 15 billion spent on healthcare annually, as it is characterized by a cycle of remission and disease flares (Mehta, 2016). Available treatments have many side effects, and some can increase patients' risk of cancer (Feuerstein, 2017). IBD's etiology is composed of both genetic and environmental factors. Recently, the latter was determined to be affected by the interactions between the immune system and the gut microbiome, a community of commensal bacteria located in the digestive tract (Ananthakrishnan, 2017). It is unknown if the effect of diet is altered by the type of microbiome present.

Review of Literature

The Gut Microbiome

The gut microbiome is linked to the etiology and pathogenesis of many diseases, including Inflammatory bowel disease. A single microbiome is a unique collection of bacteria. The function of a normal microbiome is to aid in the digestion of food, regulate the immune system, and produce vitamins. The diversity of the microbiome plays a significant role in the ability of the microbiome to complete its function efficiently and effectively. The microbiomes of IBD patients were found to have decreased microbial diversity when compared to healthy patients (Carding, 2015). Dysbiosis, or microbiota with a change in population, diversity, or stability, is a characteristic of many patients with IBD. In studies using twins and/or siblings, it

was found that the dysbiosis was not a result of IBD; instead, it was found in individuals before they developed active disease (Hedin, 2017). This showed that dysbiosis was not a symptom caused by IBD, instead, it was shown to be a possible part of the cause of the disease. Supporting studies have shown that colitis (inflammation) severity and frequency is decreased or absent in germ-free mice, which suggested that the microbiome triggered the inflammation found in IBD (Carding, 2015). Additionally, a study showed that such inflammation was a result of the interactions between the gut microbiota and the immune cells found in the mucosal layer in the digestive tract (Manichanh, 2012). The evidence put forth by previous studies suggested that the microbiome was directly linked in the etiology and pathogenesis of IBD, and specifically affected the immune system.

IBD and the Immune System

Inflammatory Bowel Disease is a chronic autoimmune disease that is affected by the microbiome. It has been determined that IBD specifically affects the immune cells in the digestive tract and alters their interactions with the bacteria found in the gut microbiome. In a study, it was found that in a murine colitis model, the introduction of anaerobic filamentous bacteria, a specific subset of the bacteria in the microbiome, caused the increase of Th17 cell development (Manichanh, 2012). Th17 cells are a specific subset of T-helper cells of the immune system and have been shown to play an important role in colitis inflammation. Additionally, a second study demonstrated that the microbiomes of IBD patients increased the levels of Th17 cells. Specifically, it was found that in ex-germ free mouse models, mice colonized with the microbiomes of IBD patients had increased pro-inflammatory Th17 cells, which increase the body's immune response, and decreased RORyt+ Treg cells, which are responsible for stopping the body from attacking itself (Britton, 2019)(Figure 1).

This discovery showed that the microbiomes of IBD patients increased the number of inflammatory cells of the body, which gave evidence to the theory that the microbiome changed the immune system. Given these findings, it is clear that the immune system is affected by microbial diversity, population, and structure of the gut microbiome.

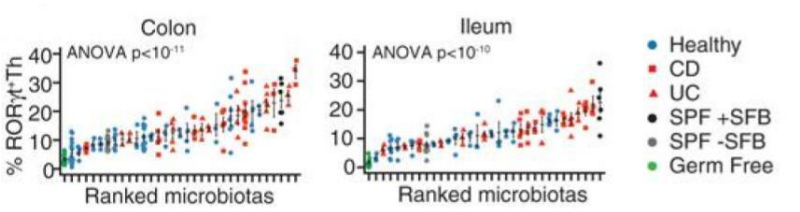

Figure 1: Colon and Ileum T-helper cells in specific mice colonized with different microbiotas (Britton, 2019)

Diet and the Gut Microbiome

One of the most well-known methods of potentially altering the gut microbiome is the modification of diet. Studies have used this method to investigate the effects of certain types of macronutrients, such as protein and fiber, on the microbiome and body. In one study, it was found that diets high in psyllium, a type of fiber, had an anti-inflammatory effect on mice treated with dextran sulfate sodium, which caused inflammation similar to that of IBD, and decreased colitis severity (Llewellyn, 2018). It was also found that diets high in casein, a protein, had the opposite effect when compared to psyllium, which caused more inflammation.

In a second study, it was found that a switch in diet from a regular mouse chow to an experimental diet comprised of different nutrient concentrations and ingredients in a mouse model caused relapses of colitis in mice (Chen, 2018). In a third study, it was found that colonized germ-free mice placed on a high sugar- high protein diet had a significant change in the structure of the microbiome within 1 day (Figure 2). Specifically, the levels of Bacilli and Erysipelotrichi bacteria were higher than those of mice on a low fat, plant protein-diet

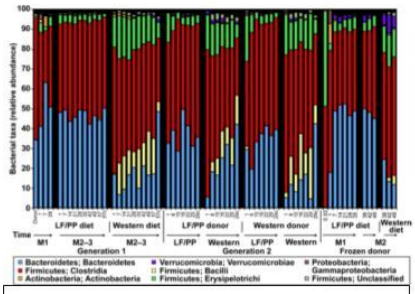

Figure 2: Taxonomic distribution of two generations of colonized mice fed either a plant protein diet or a Western Diet. (Turnbaugh, 2009)

(Turnbaugh, 2009). This result showed that the diversity increased based solely on the diet, which supported the ability of the microbiome to change due to diet. These results attested to the impact of diet on the diversity and population of the microbiome and demonstrated that diet was able to alter the microbiome in a positive manner. However, it is unknown if the effects of diet, which in turn affect the immune system and the body in general, are affected by the specific microbiome in mouse models.

Problem Statements

1. The impact of the specific microbiomes on the effects of diet high in protein in mouse models is unknown.
2. The impact of the specific microbiomes on the effects of diet high in fiber in mouse models is unknown.

Objectives

1. To determine the impact of specific microbiomes on the effects of diet high in casein by analyzing the levels of immune cells after diets are started in mice colonized with two different microbiomes.
2. To determine the impact of specific microbiomes on the effects of diet high in psyllium by analyzing the levels of immune cells after diets are started in mice colonized with two different microbiomes.

Hypotheses

1. If mice colonized with IBD microbiomes are put on diet high in casein, the diet will have a significant effect on the levels of inflammatory immune cells. This will show that the pro-inflammatory effect of a protein-based diet is unchanged by an inflammatory

microbiome, since previous research has showed that diets high in protein were successful in increasing inflammatory markers in mice with IBD (Llewellyn, 2018).

2. If mice colonized with non-IBD microbiomes are put on diet high in psyllium, the diet will not have a significant effect on the levels of inflammatory immune cells. This will show that the anti-inflammatory effect of a fiber-based diet is decreased by an non-inflammatory microbiome, since previous research has showed that non-IBD microbiomes do not increase immune cell levels, meaning the diet would not have a significant effect in decreasing the levels of immune cells. (Britton, 2019;Llewellyn, 2018).

Methodology

Role of Student vs. Mentor

Over the course of 7 weeks, during the summer of 2019, I, assisted by the mentor, performed analysis on excess murine tissue. The mentor colonized 24 C57BL/6 mice with fecal microbiome cocktails from IBD and non-IBD patients (IACUC #: LA13-00058). The mice were then placed on either a high psyllium diet or a high casein diet by the mentor for 2 weeks. The mentor provided the student with excess intestinal tissue from the mice, and the student used this tissue to prepare, garner, and analyze immune cell data using flow cytometry. The mentor also provided guidance and assistance with the execution of the methodology. All other steps reported were done by the student, such as tissue preparation, tissue digestion, and flow cytometry data analysis.

Tissue Preparation

Tissue preparation was done to prepare the tissue for digestion and staining and to remove any fecal matter or debris from the tissue. The colon, small intestine, and mesenteric lymph node (MLN) tissue from each mouse were given to the student as excess tissue from another study being conducted in the lab (Figure 3). These tissue types were used because of their high concentration of immune cells directly impacted by the microbiome. The fecal matter was removed from the colon and small intestine tissue and discarded. The small intestine was checked for Peyer's patches, small masses of lymphatic tissue, which were subsequently removed, to prevent unrelated immune cells from being seen in later steps. The colon and small intestines were then opened and cut lengthwise down the middle to expose the epithelium and muscle tissue. The inner tissue of the colon and small intestine was scraped off to remove the epithelial cells, and the tissue was placed in collection tubes with 10 mL of cold RPMI media to prevent cell death during transport to the lab. The MLNs were also placed in collection tubes after being separated from their fat coating and brought back to the lab for tissue dissociation and digestion.

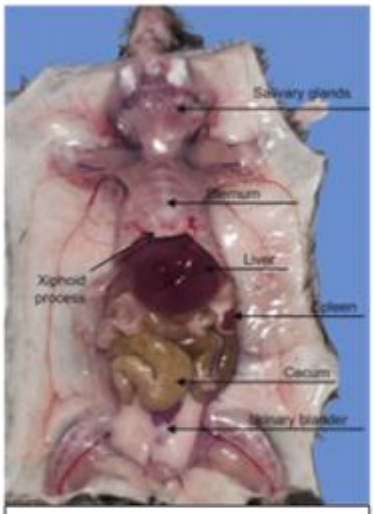

Figure 3. Open mouse internal cavity where colon, small intestine, and MLN tissue are found (ScienceDirect, 2018)

Colon and Small Intestine Tissue Dissociation and Digestion

Tissue was dissociated and digested to remove cells from the tissue and to prepare the cells for staining. Colon and small intestine tissue were transferred from the collection tubes to a second tube, which was filled with 10mL Hanks' Balanced Salt Solution (HBSS). These tubes were vortexed briefly to fully immerse the tissue in the HBSS to keep the cells in a stable environment. Then, the tissue was transferred to a third tube, containing 10 mL of dissociation

buffer. Each tube was shaken vigorously in an incubator for 30 minutes at 37°C and 110 RPM in order for the enzymes to break apart the tissue. The tubes were removed from the incubator and vortexed to prevent clumps of tissue (Figure 4). The tissue was transferred to another tube containing 20mL of cold HBSS and vortexed briefly to remove traces of dissociation buffer. Afterward, the tissue was transferred to a new tube containing a warm digestion buffer. The tissue was cut into ¼ inch pieces to increase its surface area and allow the digestion buffer to be more effective. The tubes were then placed in the incubator at 110 RPM and 37°C for 30 minutes to allow tissue digestion to occur. After the tubes were shaken, the tissue was mixed by pipetting it up and down and then pipetting it through a filter into a tube, which contained 10 mL of cold RPMI. The strainer was then washed with an additional 10 mL of RPMI to prevent cells from clinging to the strainer. The tubes were then centrifuged at 1500 RPM and 4°C for 5 minutes. The supernatant was removed by aspiration and the cells were washed in RPMI to remove any traces of digestion buffer. Finally, the cells were resuspended in 200 µl of RPMI.

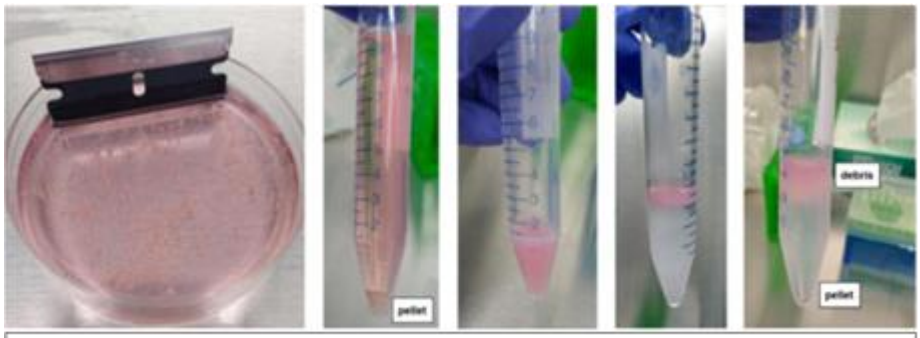

Figure 4. Example photographs of tissue dissociation (Tome-Garcia, 2017)

MLN Preparation

MLNs were prepared differently than colon and small intestine tissue due to their structure. This preparation was done to prepare the samples for staining because MLN tissue did not need to be dissociated or digested. MLNs were poured from the collection tube into a 70uM filter on a petri dish and forced through, in order to remove any fat or coating from the cells.

10mL of phosphate-buffered saline (PBS) was then pipetted through the strainer to wash out any remaining cells clinging to the filter surface. The liquid in the petri dish was transferred into a tube, and the petri dish was washed with an additional 5 mL of PBS and then transferred to the tube. Each tube was spun in the centrifuge for 5 minutes at 1500 RPM and the supernatant was discarded. The cells were then resuspended in 200 µl of RPMI and left on ice until needed for staining.

Cell Surface Staining

Staining was done to allow the flow cytometry machine to detect specific antibodies in the cells, thereby identifying each type of immune cell. 100 µl of each sample (colon, small intestine, and MLN) was pipetted twice into a 96-well plate in order to create a set of samples dedicated to myeloid cells and a set of samples dedicated to T cells. The plate was labeled with each tissue number, as well as the type of tissue per column and row to prevent confusion. The plate was subsequently spun for 5 minutes at 1500 RPM at 4◦C and the supernatant was discarded. A Zombie Aqua dye solution was made at 1:1000 concentration in PBS and 100µl of the solution were added per well. The plate was left to incubate at room temperature for 30 minutes while wrapped in foil to prevent its exposure to light. The plate was washed with 200 µl Trypsin with EDTA solution (FACS) buffer to remove excess Zombie Aqua dye solution from the plate. The surface antibody mixture was made using several antibodies and diluted in PBS. The surface antibody mixture contained several fluorophores, which were bound to antibodies that would detect each specific protein on the surface of the cell (see Table 1).

The specific fluorophores allowed the flow cytometry machine to register the volume of each marker for the cells, thereby allowing it to detect the specific subsets of cells. Then, 100 µl of surface stain mixture was added to each well and the plate was incubated in a 4◦C fridge for 20 minutes protected from light. The plate was then washed twice with 200 µl FACS buffer each time to remove excess surface stain mixture after staining had been completed.

Table 1. (A) T Cell Surface Stain Markers and their corresponding Fluorophores used for this study
(B) Myeloid cell Surface Stain Markers and their corresponding Fluorophores used for this study
Table by L. Kitts

A

T cell Marker	Fluorophore
CD45	APC-Cy7
CD4	PerCP-Cy5.5

B

Myeloid cell Marker	Fluorophore
CD45	APC-Cy7
MHCII	PB
CD11c	PE-Cy7
CD64	PE
CD11b	PerCP-Cy5.5
Ly6C	FITC
Ly6G	Alexa Fluor 700

Fixation and Intracellular Staining

Fixation was done to prevent cell degradation before the samples were analyzed while the intracellular staining was done for the T cell panel to look at the antibodies within the cell. 100 µl of fixation buffer was added to each well of the plate and left overnight in the 4◦C fridge protected from light to prepare the cells for intracellular staining and prevent cell degradation. The myeloid samples were then moved from the original plate to another 96 well plate to prevent cross-contamination. The original plate with the T cell panel was washed 3 times with 200 µl FACS buffer to remove any remnants of the fixation buffer. Then, 200 µl of permeabilizing buffer was added to each well of the T cell plate to prepare the cells for intracellular staining, and the plate was spun in the centrifuge for 3 minutes at 2000 RPM. The supernatant was discarded following the centrifuge spin. 50 µl of intracellular antibody mix was added to each well of the T Cell Panel (see Table 2). The intracellular antibody mix

acted to identify the specific marker proteins within the T cells and allowed the flow cytometry machine to detect the subsets of T cells associated with each protein.The plate was then incubated in the 4°C fridge for 30-45 minutes protected from light to allow the intracellular mixture to stain the cells. Finally, the plate was washed three times with 200 µl permeabilizing buffer and then resuspended in 200 µl of PBS.

Table 2. T Cell Intracellular Markers and their corresponding Fluorophores used for this study Table by L. Kitts

T cell Marker	Fluorophore
FoxP3	PE
RORγ+	APC
GATA3	BV421
Helios	FITC

Flow Cytometry

Flow cytometry was performed for each sample by the designated supervisor using the Aurora Flow Cytometer to quantify the levels of each type of cell in the T cell and myeloid cell panels based on either positive or negative fluorophore markers. The positive markers showed a reaction to the presence of each fluorophore, which the flow cytometry machine would record. The results were then analyzed by the student using the software FlowJo.

Statistical Analysis

Once the results were analyzed using FlowJo, the data was graphed using GraphPad Prism and DataClassroom based on the percentages of each parent category of a cell type. Statistical significance was determined by error bars and their overlap, in addition to a student's t-test to determine p values. The statistical test was determined based on DataClassroom's algorithm.

Results and Discussion

Colon Cells

Colon tissue was analyzed to determine the levels of many key T cell subsets to determine the effect of the microbiome on previously studied diets. In the colonic Th17 cells, there was a trend of increased Th17 cells in the mice which received the high psyllium diets for

both 099B microbiota and the m87 microbiota, which was shown in previous research (Llwellyn, 2018). However, no significant change was seen between the two microbiotas ($p > 0.05$). Additionally, in the colonic tissue, it was found that there was an increased amount of RORgt+ Treg cells in the 099B colonized mice on the high psyllium diet ($p < 0.05$). The RORgt+ Treg cells did not show significant change between the 099B microbiota and the m87 microbiota across either of the two sets of trials ($p > 0.05$). This meant that both diets had the same effect, which was different than previous research. Also, the colonic FoxP3+ Treg in mice colonized with m87 microbiota that received high psyllium diet increased (Figure 5). This result was inconsistent with previous studies, which showed that FoxP3+ Treg cells did not increase in mice that received the high psyllium diet. Overall, the diets did not show the clear differences which were reported in a previous study because the colonic T cells showed very few significant differences between the two diets in either microbiome (Llewellyn, 2018). The lack of the distinction in T cell subsets based on diet is concluded to be a result of the microbiomes that were introduced into the mice. This is because, unlike Llewellyn in 2018, there were different microbiomes used in this study. Because of this, the results provide support for the hypothesis that the microbiome affected the impact of the two experimental diets.

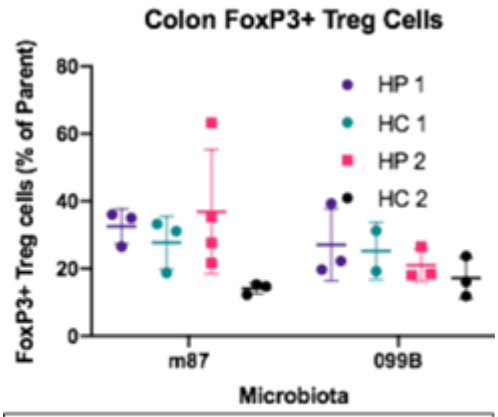

Figure 5: Colonic FoxP3+ Treg Cells for both diets (High Casein, High Psyllium) and both microbiotas (m87, 099B)

MLN Cells

In the mesenteric lymph node (MLN) tissue, there was almost no change between the two diets when the levels of T cell subsets were analyzed to determine the effect of the microbiome on the

two experimental diets. However, this was not the case for the FoxP3+ cells. In the mice colonized with 099B microbiota, there was a significant difference between the levels of FoxP3+ Treg cells between mice on the high psyllium diet versus those on the high casein diet ($p < 0.05$). Specifically, the levels of FoxP3+ Treg cells were higher in the high psyllium diet than in the high casein diet (Figure 6).

Additionally, it was seen that the RORgt+ Treg cells in the MLN tissue were not the same between the m87 microbiota and the 099B microbiota. In the m87 microbiota, the data was varied, and Treg cells were more concentrated than in the 099B microbiota (Figure 7). In the 099B microbiota, the data was very similar, and there was no statistical significance found between the two diets.

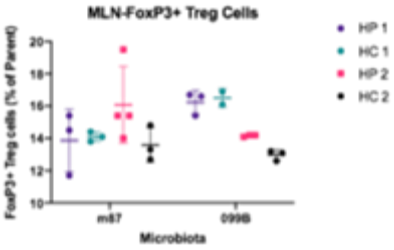

Figure 6. MLN FoxP3+ Treg Cells
Figure by L. Kitts

The significant change of the RORgt+ Treg cells in the MLN tissue between the two microbiotas indicates an effect by the microbiome type to change the impacts of the two diets. Throughout the cell populations, the MLN tissues had more varied levels of T cell subsets in mice colonized with m87 microbiota than with 099B. This is consistent with past studies, which showed that 099B microbiota is less inflammatory than m87 microbiota, which was originally colonized from a patient with Inflammatory bowel disease (Britton, 2019). However, these findings are inconsistent with previous research regarding diet, like Llewellyn in 2018. The difference in results is attributed to the change in methodology to include the two

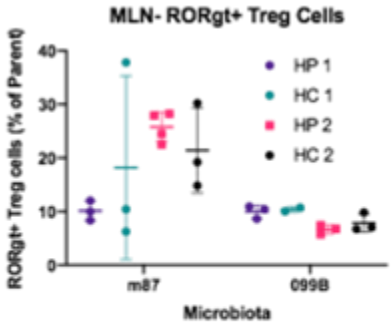

Figure 7: MLN tissue percentage of RORgt+ Treg cells in both diets and microbiotas.

different microbiomes. The results for the MLN tissue T cell subsets showed no difference between the two diets in the less inflammatory microbiome.

Small Intestine Cells

There was an unexpected result seen in the small intestine tissue samples, which prevented any conclusions from being made about the tissue. In all of the mice on the high psyllium diets, there were no live CD45+ cells (Figure 8). CD45+ cells refer to all immune cells, so this was detrimental to the collection of data for the purposes of the study. The small intestine tissue regardless of diet or microbiome has been shown to have immune cells in previous studies. This result was most likely due to the preparation of tissue samples after tissue collection. It was most likely not an effect on the small intestine tissue directly since the mice were all healthy and alive before sacrifice, and they can not live without CD45+ cells. In summary, the small intestine tissue showed a surprising result of having almost no live cells which prevented the interpretation of data relating to the small intestine tissue.

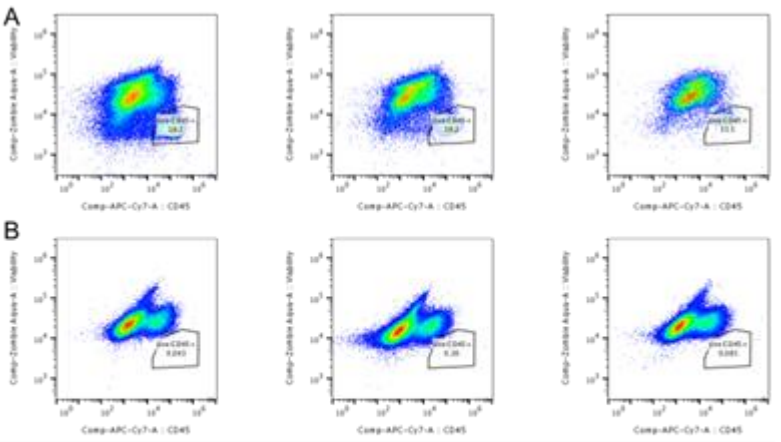

Figure 8: (A) Flow cytometry analysis graph on mice on high casein diet
(B) Flow cytometry analysis graph of mice on high psyllium diet
Picture by L. Kitts

Implications and Applications

The application of this study is very specific. As Inflammatory bowel disease research transitions to research regarding the microbiome, this study is a step toward more knowledge and can help lead to possible treatments involving diet and the microbiome for IBD. Research about the microbiomes specific to IBD patients can be applied for specific drug development or other

treatments for IBD. Additionally, this research can be applied to other diseases that are affected by the microbiome. Because many diseases have been linked to the gut microbiome, this could be used when creating and testing treatments for those diseases as well in order to test the efficacy of the treatment in microbiomes colonized from patients.

Future Research

Future studies planned in this field of research should focus on other microbiomes for use in colonizing the mice and examining their effect with the same diets. The tissue of these mice would then be prepared, digested, stained, and analyzed in a similar manner to this study to determine the effects. Specifically, the microbiomes of the two sub-diseases within IBD, Crohn's Disease and Ulcerative Colitis, would be ideal candidates, due to their previously researched differences. A study looking at this would also help with the understanding of the differences between Crohn's Disease and Ulcerative Colitis in terms of treatment. Further research would be important to help understand the effects of different microbiomes and how the diets are affected by them, to further the study of IBD and its possible treatments and their efficacy.

Conclusion

Currently, there is a lack of knowledge about the interactions of the microbiome in the gut with diets high in fiber or protein. The objective of this study was to determine the effect of two experimental diets on the inflammation linked immune cells in mice colonized with IBD microbiomes and non-IBD microbiomes. The study was conducted through the use of excess tissue from mice colonized with either microbiome which received diets high in psyllium or casein. The tissue was prepared through tissue digestion, stained, and analyzed through flow cytometry. The results showed that the microbiome and its properties had an impact on the efficacy of the diets high in fiber or protein with regard to the immune system. These results may

help explain why the use of diet as a treatment is not an exact science and has had different results in different patients. Additionally, they can be applied to drug development specific to the microbiome and IBD, and possibly other diseases. This study showed that the microbiome was able to affect treatments to inflammation and increased the understanding of the gut microbiome.

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