THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

DIET AND ANTIBIOTIC INFLUENCES ON MICE INDUCED WITH DSS COLITIS AND THEIR GUT FLORA

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Spring 2013

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Veterinary and Biomedical Sciences
with honors in Veterinary and Biomedical Sciences

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Abstract:

Inflammatory bowel disease causes a variety of problems in patients. The most obvious is the serious degradation of the intestinal tract. It has been shown that specific diets have ameliorated as well as aggravated the intestinal lining. The specific mechanism and reason for this exacerbation is relatively unknown, however there are notably two possibilities: diet influences gut flora, promoting helpful bacteria or decreasing harmful ones; or that the diet has an impact upon the host's immune system, compromising the host. First, the diets' (specifically types of carbohydrates) effects in mice induced with a mouse model of inflammatory bowel syndrome called dextran sulfate sodium (DSS) was investigated. To understand the central cause of inflammation, the successive study utilized antibiotics to alter the composition of the bacteria flora in the gut and examine how it affects symptom severity of DSS colitis. Through the analysis of weight change, fecal DNA, as well as colon length changes, the correlation between gut flora can be studied. Three diets were used: Chow (CD, LabDiet 5001), Harlan Teklad (TD, TD96348), and a purified diet (PD) (made in the laboratory). There are multiple differences in the diets' composition. The CD diet contained the greatest diversity of fiber. The TD diet, with significant makeup of starch, lactose, and fiber (cellulose) had the most negative effects on the mice induced with DSS. Conversely, the mice fed PD, with dextrose and sucrose, and less fiber, proved to perform better all around, while mice fed TD performed significantly worse. This was determined via relative weight changes, as well as colon shrinkages. After this, antibiotics were introduced in the mice's water with either TD or PD. The influence of antibiotics had a greatly positive consequence on mice given both types of diets, highly outperforming their

control counterparts. This promotes the theory that the gut flora largely controls the inflammation of the gut.

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Acknowledgements

I would like to thank all of the undergraduates who helped me throughout this entire experience, including Austin Corrs, Victoria Holden, Nina Schumack, Laura Rust, Abby Urraro, and Shelley Wilke.

I would also like to thank Rhonda Smith and Jot Hui Ooi for their help in explaining and allowing me to incorporate the DGGE samples and the bacteria sequencings for both experiments.

Lastly, I wish to thank Dr. Cantorna who has helped me throughout this entire process and throughout my college Career.

Introduction:

Inflammatory bowel disease (IBD) is an idiopathic disease. Two such types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Within the United States alone, over 700,000 people have CD and over 1 million have IBD. Similarly, it has been shown that CD, as well as ulcerative colitis is more prevalent in more developed countries, with the most cases within the United States.[1] These people suffering with UC and CD experience symptoms that range from abdominal cramps and pain, bloody diarrhea to more debilitating problems like fever and intestinal wall ulcerations. As the patients' intestinal absorption becomes compromised from constant damage to its walls, their body's absorption of important nutrients become significantly decreased. Researchers have discovered that certain foods may worsen IBD symptoms while others have been shown to improve them; some have even suggested undergoing strict nutrition regiments. These include the promotion of breastfeeding in babies showing IBD-like signs, as well as the supplementation of polyunsaturated fatty acids, butyrate, and glutamine to lessen the inflammatory response in the gut.[1] There are a variety of solutions that doctors may prescribe. These treatments could be anywhere from diets, to antibiotics, to corticosteroids, immunomodulators, or other biologic therapies.[2] These have all been shown to keep episodic flare-ups to a minimum. Unfortunately, this wide range of possible treatments suggests that researchers do not truly understand the underlying problem. Is it nutrition, immunological problems, or gut flora that causes the most influence in these patients; or is the problem a combination of the above?

Diet

Nutrition has a major role in the cause of IBD flare-ups, as well as their remissions. Perhaps most influential are the variety of carbohydrates within diet. These can either exasperate or improve the IBD disease state.[3] Some people with IBD become more sensitive to some carbohydrates. One such study shows the association of lactose sensitivity in people with IBD. Over 70% of patients with IBD had a lactose sensitivity.[4] The digestibility of carbohydrates also plays a factor. One study has shown that the digestibility of the carbohydrate chains is directly proportional to the symptomatic expression in patients suffering with IBD. [5] People who consumed simpler carbohydrates had less volatile fatty acids, specifically butyrate, which helps maintain and repopulate colonocytes within the colon. [5] Conversely, those who ate less digestible carbohydrates showed an increase in these fatty acids. [5] In a piglet model lower digestible, larger carbohydrates have beneficial effects on IBD symptoms.[5] Similarly, some scientists suggest that an increase dietary fiber has tremendous impact in keeping IBD in manageable state of remission and actually shows better performance within patients struggling with IBD. [6]

Microflora

Relatively recently, people have started describing the bacterial composition of the gut as having a large impact upon our nutrition and health throughout our body. These health effects can be discovered throughout our body, even directing our nervous system.[7] Scientists have shown that gut flora may directly influence autoimmune demyelination, showing that the bacteria have more influence over our bodies than imaginable in the past. [8,9] Similarly, antibiotics have been used as a therapy for IBD patients in order to modify the

gut flora, which has proven to have beneficial consequences. The real question, however, is why have antibiotics been shown to improve IBD symptoms. Specifically, what bacteria are we destroying and keeping alive that helps us keep IBD in remission. On the other hand, perhaps by destroying certain bacteria, we are able to acquire the nutrients that are needed to fend-off IBD symptoms.

It is important to analyze the correlation between nutrition and symptomatic expression in mice because one of the large components that varies gut flora among hosts involves the quality, quantity, and specific make-up of our diets. A person's long-term diet creates a specific niche for the organisms within our gut to live. Specific species that take advantage of the nutrients that we supply will outperform and therefore a larger percentage of the gut than those that are not as well suited. [10] Different types of carbohydrates influence the gut flora make-up. Because bacteria are more efficient at breaking down fiber, other bacteria may not have the enzymes needed to convert the chains of sugars into dimers and monomers.

Immune Function

Though bacteria within the gut plays a role in IBD, the immune system should not be overlooked as a contributing factor that exacerbates and/or ameliorates IBD symptoms. A major immune regulator in the body is located adjacent to the gut. As the gut is essentially contiguous with the outside flora, the body must constantly fend off the pathogens within the body and around these mucosal membranes, which is essentially contiguous with the outside environment. Recently, people have been researching if in fact the flora within the gut directly controls our immune system.

It has been discovered recently that the immune system malfunctions, causing flare-ups in IBD patients. The immune system normally has the ability to differentiate between foreign pathogens and nutritious material. However, in people with IBD, the immune system sees non-self antigens within intestinal cells as dangerous, which elicits an immune response. Immune cells attack these "infected" intestinal cells, as well as triggering the migration of more white blood cells, creating chronic inflammation.[11]

Summary

The significance of IBD cannot be understated. As over one million people are suffering with this disease, the cause and its treatments are crucial. A large amount of research has been devoted to diet and how it affects IBD symptoms. It has been found that carbohydrate components within a diet can have varying effects upon IBD severity. Looking deeper into this discovery, scientists have began looking into the possibility of gut bacteria in both the exacerbation and amelioration of IBD symptoms, which may be moderated by diet, specifically carbohydrate composition. Similarly, the immune system and how it responds to the varying conditions within the gut has proved to play a role in IBD. As such, both bacteria and immune system influences must be studied to determine the precise roles that each of them have in symptom expression and disease status.

Hypothesis

We hypothesize that the effects of dietary dose on the experimental colitis is thought to alter the composition of the gut flora, thereby affecting symptomatic expression.

It is not to be overlooked, however, that it has been shown that diet indirectly affects the immune system, which may have also contributed to a difference in disease status.

Materials and Methods:

Animal Model

A model that emulates a short-term IBD in mice is obtained via the chemical dextran sulfate sodium (DSS). The disease status in this model is marked and directly proportional to the weight loss in the mice, as well as the hyperplasia of the colon: the more weight loss and smaller the colon, the sicker the mouse. The DSS model is also self-limiting, meaning that the mice will naturally recover from their disease state.

Diets

The preliminary experiment was performed to determine the effects of solely the diets upon the symptoms of DSS colitis. The diets used in the experiment are Purified Diet (PD), a diet that is constructed within the lab based on basic dietary and nutrition needs of the mice, as well as a Teklad White Pellet diet (TD.96348, abbreviated TD with ingredients seen below), a diet produced by Harlan laboratories, and mice chow diet (CD), which is the standard diet given to mice in laboratory settings. The TD had only one source of fiber (cellulose) and three sources of carbohydrate (lactose, sucrose, and starch). PD had less fiber (3% instead of 5%) and two sources of carbohydrate (sucrose and dextrose). CD has six sources of carbohydrates (dextrose, fructose, glucose, lactose, starch and sucrose) and is composed of 5.1% crude fiber.

Table 1: Comparison of Diets with Relevant Nutrients			
	Purified Diet (PD)	Teklad .96348 Diet (TD)	Chow Diet (CD)
Total Carbohydrates (%)	**73.2**	**67.5**	**58**
Dextrose (%)	44.1	-	44.1
Glucose (%)	-	-	0.2
Fructose (%)	-	-	0.3
Lactose (%)	-	15	2
Starch (%)	-	26.4	31.9
Sucrose (%)	21.7	20	21.7
Total Fiber (%)	3	5	7
Cellulose (%)	3	5	-
Crude (%)	-	-	7
Protein (%)	21.2	19.9	28.5
Fat (%)	5.6	12.7	13.5

(Calculations were done by Jot Hui Ooi)

Antibiotics

In the second portion of the experiment, half of the mice were given antibiotic treated water to alter gut flora. This was accomplished with a cocktail of antibiotics. The four antibiotics used were ampicillin, vancomycin, neomycin sulfate, and metronidazole. Each of these antibiotics has a unique function that eliminates different types of bacteria. Ampicillin is a molecule that acts as a competitive inhibitor with an enzyme called transpeptidase, an integral protein in bacterial cell wall synthesis, specifically Gram-negatives. Vancomycin is similar to ampicillin in that it inhibits cell wall synthesis, but through a different mechanism; vancomycin also kills mostly Gram-positive bacteria. Neomycin sulfate interferes with protein synthesis, binding to the 30S subunit of bacterial ribosomes. Lastly, metronidazole creates sulfide bonds with bacteria enzymes that help deactivate them, disrupting many processes and resulting in cell death. All of these antibiotics in concert with each other destroyed most of the bacteria within the mouse gut

Table 2: Antibiotic Cocktail
Ampicillin 1 g/L
Metronidazole 1 g/L
Neomycin Sulfate 1 g/L
Vancomycin 500 g/L

Colitis Severity

Mice suffering with DSS induced ulcerative colitis exhibit predictable and measurable symptoms. These symptoms portray the severity of their illness. The irritation and aggravation of the gut lining directly prohibits absorption of nutrients, resulting in weight-loss proportional to the severity of inflammation. If the colon becomes irritated enough, it may start bleeding internally, with blood apparent in the feces. The irritation also results in a large shrinkage of the mice's colon, also directly proportional to degree of colitis within the mouse. Serum, histopathology, RNA, MLN, and pH of colon contents will also be collected for further analysis. If a mouse lost 20% of their body weight any time throughout the experiment, that mouse was sacrificed.

DGGE

To separate and identify bacteria from the feces, denaturing gradient gel electrophoresis (DGGE) was used. DGGE operates similarly to normal gel electrophoresis in that small segments of DNA travel further through the gel than large segments. However, another dimension is introduced: a denaturing gradient. The combination of heat and denaturing enzymes break the cytosine to guanine bonds in DNA.

Experimental Design

Groups of 5 mice were fed each diet for 2 weeks. After these two weeks, all of the mice were given 2.5% DSS induced colitis via their water. The mice were given this DSS water for 5 days and then given tap water for 7 days for recovery, after which they were sacrificed (at day 12). The mice were weighed every day from the start of 2.5% DSS.

Significance:

The unpaired T-test was used using Microsoft Excel for statistical analysis of the data. A p-value of less than 0.05 was used to determine significance.

Results:

Diet effects on DSS colitis

In the preliminary experiment a large difference primarily between the TD and PD mice were seen. As seen in Figure 1, by day 6 of the experiment, all of the TD mice lost more than 20% of their body weight and were sacrificed. Conversely, all of the PD mice survived and regained their lost body weight by 10 days post DSS. Further, the PD mice only lost 5% of their original body weight. The CD mice lost about 15% of their body weight, and failed to fully regain their body weight by day 12 post DSS. The body weight of the mice at day 6 were significantly different between TD and PD fed mice.

Similarly, the PD mice had an average colon length of 90 mm at day 14, while the CD mice had average colon that were significantly similar. However, the CD mice showed no significant differences in their colon compared with the PD mice, despite the large weight difference. The colon lengths of the TD mice were not compared to the mice given the other two diets because they did not reach the end of the recovery time (day 14). The TD mice were unable to survive past day 6. One dipped below 80% body weight after day 5, while the other four lost 20% body weight at day 6 (Figure 3).

Figure 1: Changes after induction of inflammation in mice fed CD, TD, and PD.

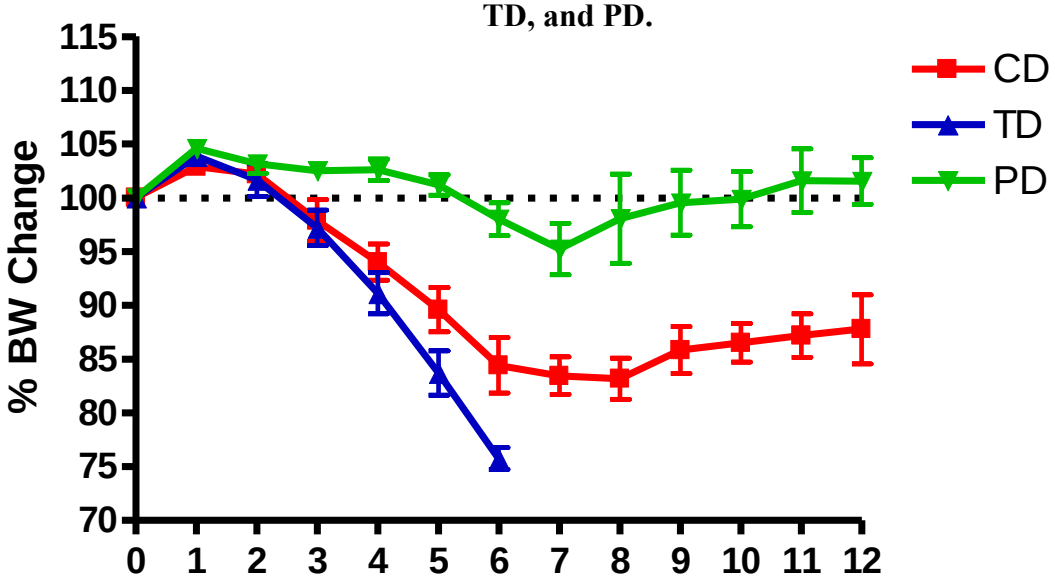

Figure 1 | Weight changes of the mice given different types of diets. Red represents the Chow Diet (CD), blue represents the Teklad Diet (TD), and green represents the Purified Diet (PD). DSS treated water was given at Day 0 until Day 5, after which regular water replaced the DSS water.

Figure 2: Colon Lengths of Mice Fed CD and PD at Day 12

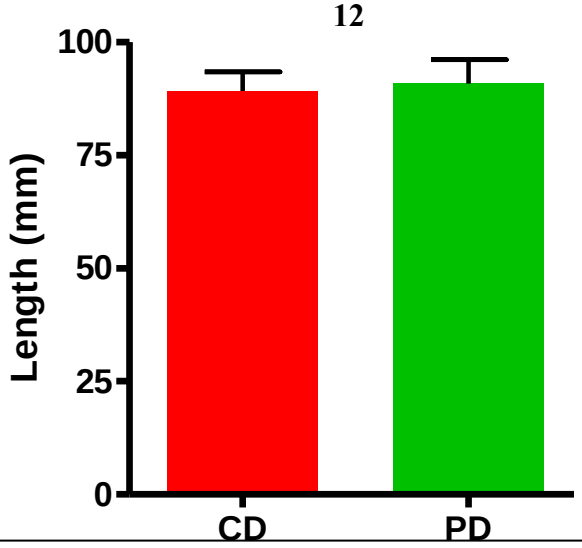

Figure 2 | Colon lengths of the mice after sacrificing. The TD diet is not present because they did not reach the designated sacrificing day (day 12).

Figure 3: Survival Curve of PD, TD, and CD Mice Following DSS Colitis Induction

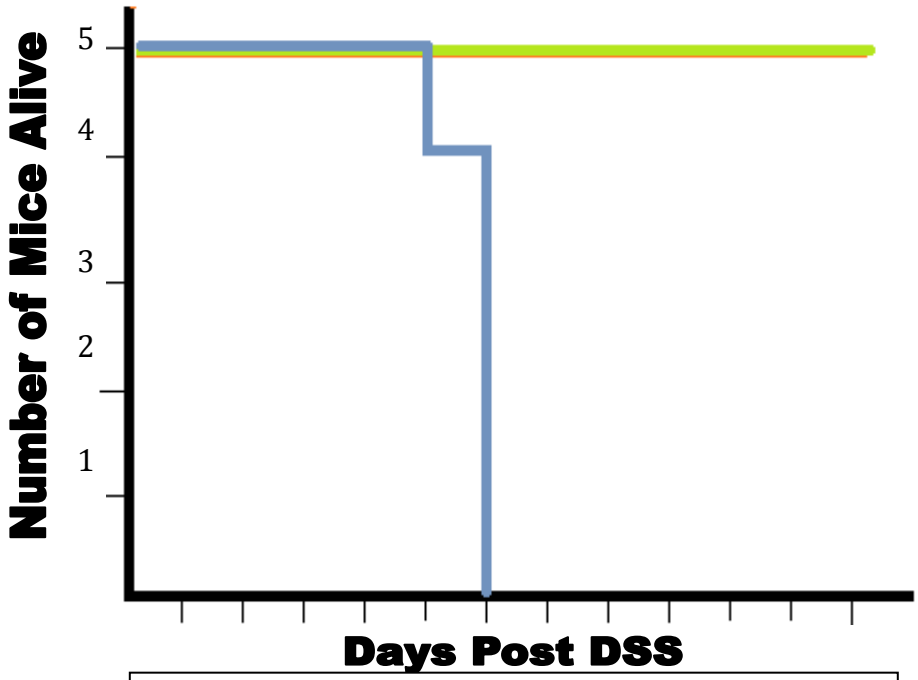

Figure 3 | Survivability of mice induced with DSS colitis on different diets. Blue represents TD. Green represents PD and orange represents CD.

Effect of diet of bacterial flora

The feces of the mice were collected and DGGE was run to compare the microflora in the gut. As a result of the diet changes, the data indicated that the diets significantly changed the gut's diversity (Figure 4). The sections in red show the banding differences in the DGGE gel. Bacterial sequencing showed a 30% difference in the amount of *Clostridium* species (34% in the PD mice as opposed to 64% in the TD mice).

Figure 4: DGGE and Bacterial Analysis on Feces in Mice fed TD and PD

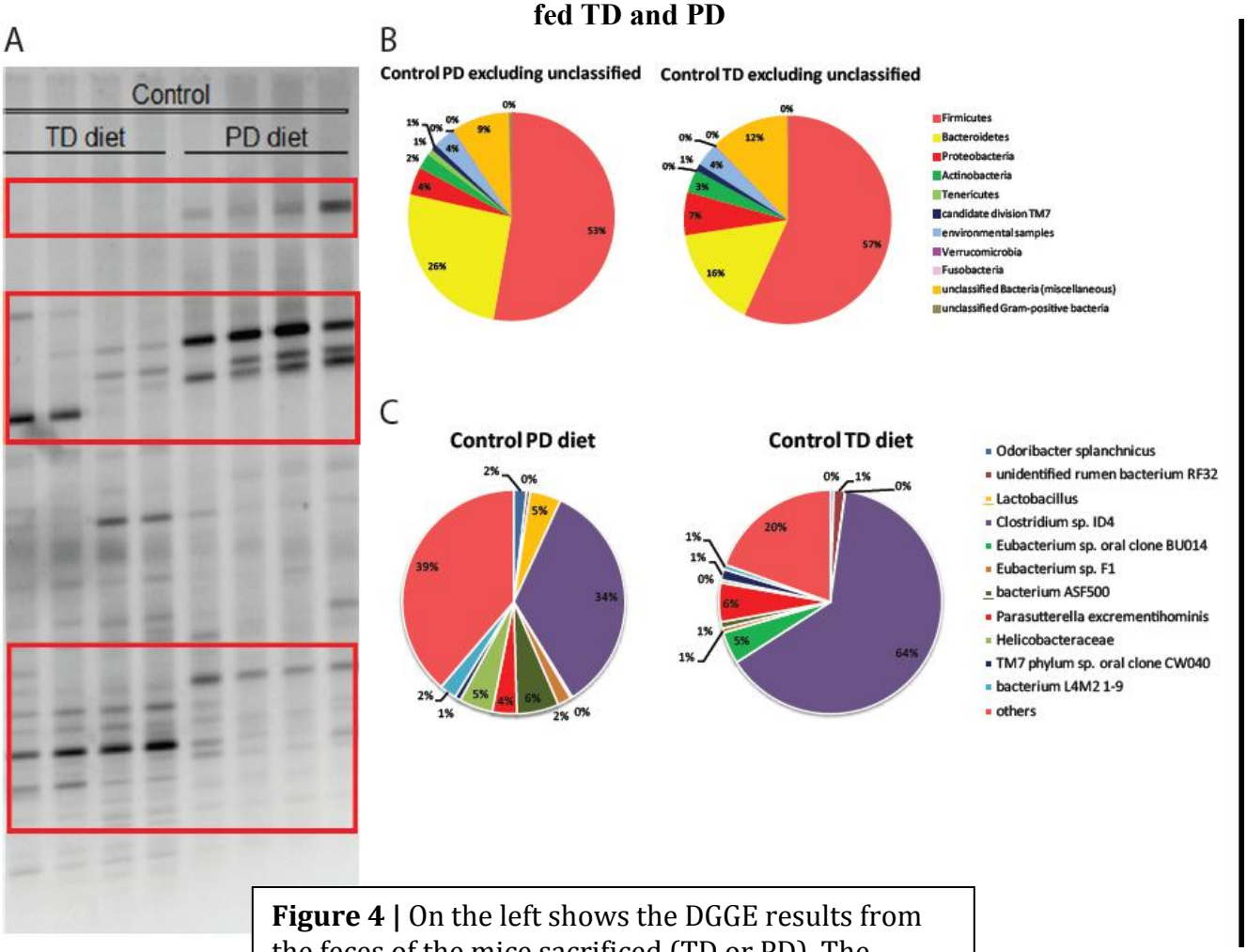

Figure 4 | On the left shows the DGGE results from the feces of the mice sacrificed (TD or PD). The banding differences are shown within the red boxes. On the left shows the bacterial sequencing of the DGGE. (The DGGE and sequencing were performed by Rhonda Smith and Jot Hui Ooi)

Antibiotics ameliorates DSS colitis.

Antibiotics (AB) treatment suppressed the development of DSS colitis regardless of diet. Four out of the five TD mice died before day 9 of the experiment, and one of the TD treated mice had 80% body weight, yet recovered after day 11. The AB mice overall performed better than their control counterparts. One of the PD mice and TD/AB mice was sacrificed due to 20% weight loss on day nine of the experiment, while the TD/AB and PD/AB mice all experienced very little deterioration in their health status. On day 8, the weight differences between TD and TD/AB showed significant difference with p-value equal to .0263. Similarly, the mice fed PD diets overall performed better than their TD diet counterparts, with the PD/AB outperforming TD/AB, and PD outperforming TD.

This trend continues when looking at the colon length post-experiment. Looking at Figure 5 and 6, the TD mouse's colon lengths were much shorter than those of its AB counterparts, a colon length of 70 mm, compared to an average of 92 mm colon. Interestingly enough, the TD/AB mice had longer colon than any of the other groups. Expectedly, however, the PD mice had longer colon lengths than TD mice: about 15% longer. Similarly, on average, the PD/AB mice's colon was longer than its water-fed counterparts.

As in the preliminary experiment, feces were collected and used to run a DGGE for the antibiotic experiment. According to Figure 7, the results showed that there was a variance in the bacteria flora between the PD and TD mice given antibiotics. There is a presence of *Tenericutes* and *Ureaplasma* Phyla in the feces of PD/AB mice, shown in the bacterial sequencing, while neither of these families is present in TD/AB mice. Further, there was much less variety in both TD/AB and PD/AB mice compared to their plain water

counterparts. This suggests that the antibiotics worked in either eradicating the deleterious bacteria or promoting the ameliorating ones.

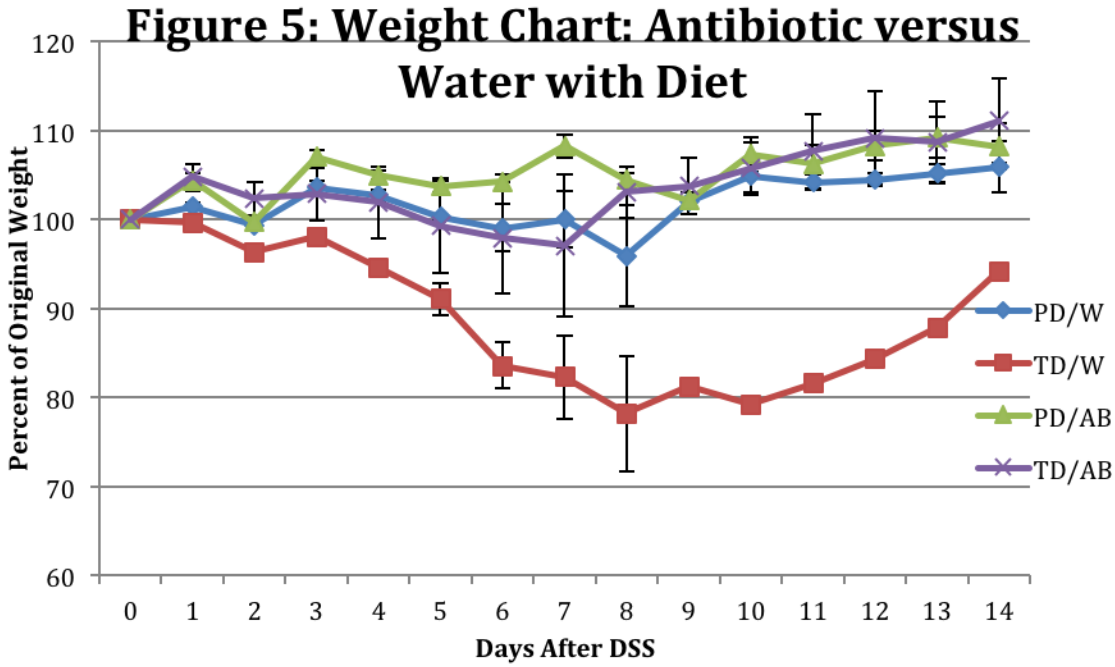

Figure 5 | Weight changes of the mice given different types of diets and presence of antibiotic treatment. Red represents the Teklad Diet with regular water (TD), blue represents the Purified Diet with regular water (PD), purple represents Teklad Diet with antibiotic treated water (TD/AB), and green represents the Purified Diet with antibiotic treated water (PD/AB). DSS treated water was given at Day 0 until Day 5, after which regular water replaced the DSS water.

Figure 6: Colon Lengths of Mice Fed PD and TD with the Addition of Antibiotic Water

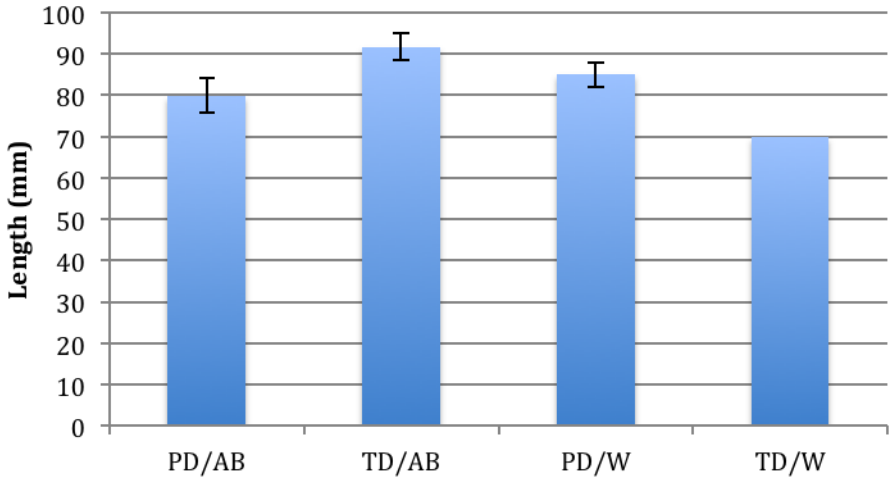

Figure 6 | Colon lengths of the mice with different variables in diet or antibiotic treatment after sacrificing at Day 14. There was no significant difference between the AB given mice and their water counterparts independent of the diet.

Figure 7: DGGE and Bacterial Analysis on Feces in Mice fed TD and PD with the Addition of Antibiotic Water

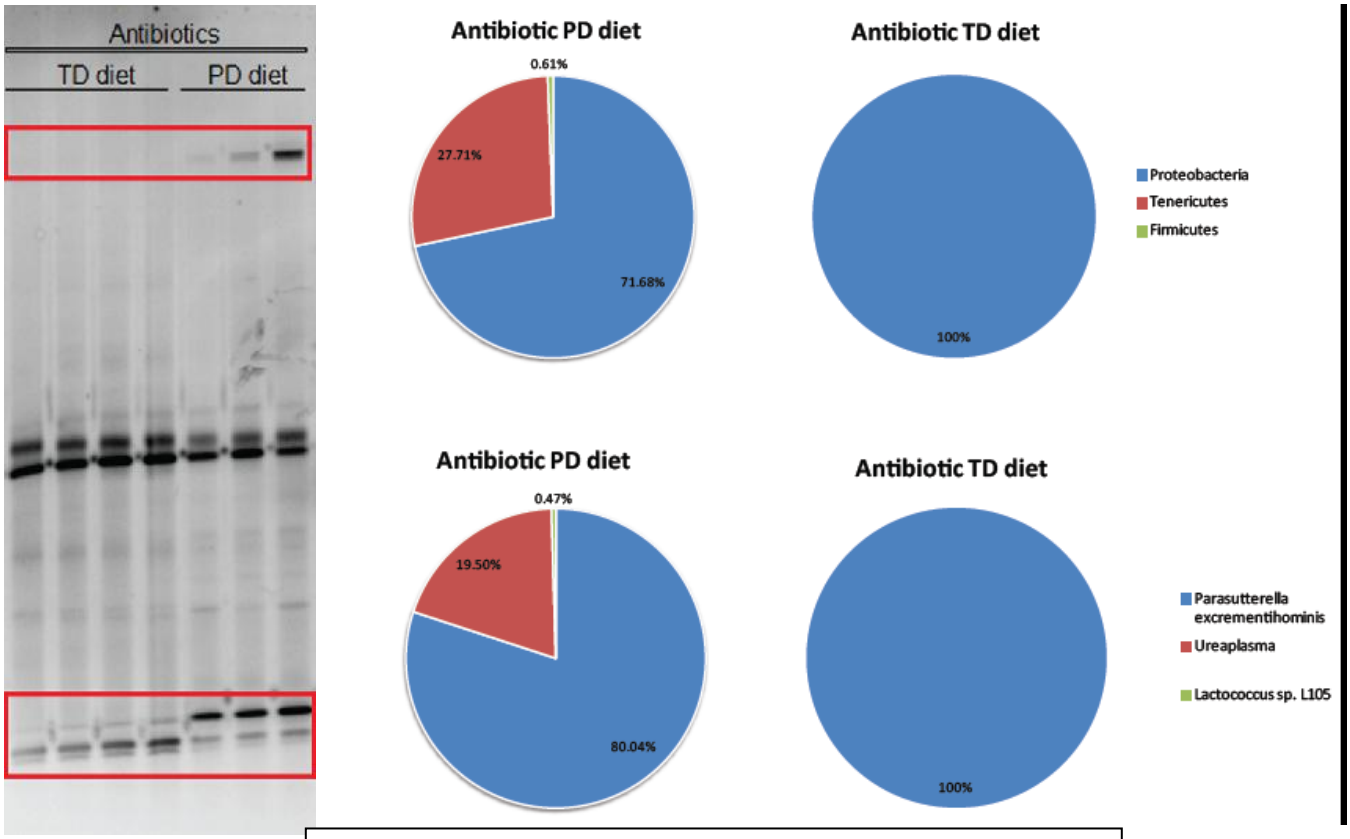

Figure 7 | On the left shows the DGGE results from the feces of the mice sacrificed (TD/AB or PD/AB). The banding differences are shown within the red boxes. On the left shows the bacterial sequencing of the DGGE. (The DGGE and sequencing were performed by Rhonda Smith and Jot Hui Ooi)

Discussion

The preliminary experiment allowed us to first look how dietary variables can have an impact on mice induced with the DSS model of colitis. The differences among the three diets were mostly concerning the carbohydrates. As expected, with different carbohydrate amounts and components, the mice's flare-ups with DSS had varying responses and symptomatic expression. The mice given the PD significantly outperformed the mice given the other two diets. The mice that performed the worst were those fed TD. All of the TD mice lost over 20% of their body weight before or at day 6 of the experiment, and therefore mercy sacrificed. The mice fed CD performed worse than the PD mice and better than the TD mice, fully recovering and gaining all of their weight back by the end of the experiment. This trend extends to the colon lengths. In the mouse model of IBD, the size of the colon mimics the disease state of the mouse. If the colon length shrinks significantly, the mouse is under tremendous stress from the colitis.

Because PD and TD utilized the same type of fiber (cellulose) with a very small difference in relative use (35g/kg in PD and 50g/kg in TD), the largest difference in dietary composition between the two extremes (the TD and the PD) was the presence of lactose in the TD and the lack there of in the PD. This seems to suggest that either the mice were lactose intolerant and unable to digest the lactose, causing irritation within the gut. Alternatively, the bacteria species in the TD fed mice caused a worse disease-state. This finding suggests that although the exact mechanism is unknown, antibiotics greatly ameliorate DSS induced colitis in mice independent of diet.

Looking at the identification of the bacteria found in the feces of the PD and TD mice (Figure 4), The highest percent of bacteria including unclassified data for the TD diet was

Clostridium, 64% of the total composition of the gut, compared to 34% for the PD mice. These results are consistent with and support the findings that many IBD patients become infected with a *Clostridium* species, some often succumbing to the infection and its toxins.[11]

The increase in *Clostridium* genera may in fact be a direct result of the usage of lactose in the diet. Similarly, since there are just a larger proportion of these bacteria within the mouse in TD, the higher cellulose content in the TD fed mice may have also achieved this effect. This is inconsistent with most findings, as fiber has been discovered to have ameliorating effects on those suffering from IBD.

Treating the mice with an antibiotic cocktail in their water supply improved the disease statuses of the mice dramatically, independent of the diet they were given. Interestingly enough, the PD/AB mice almost showed no sign that they were at all induced with colitis. They did not dip below their original body weight throughout the entire experiment. There was significant difference between the TD mice and the TD/AB mice, a p-value of less than .05. Almost all of the TD/AB mice were able to maintain the 80% body weight they needed to avoid sacrificing, while only one TD survived until the end of the experiment. This trend was also seen in the dissection of the mice, where the TD mouse's colon length was significantly less than the average of the TD/AB mice's colon. Likewise, the antibiotics destroyed all of the *Clostridium* species, the pathogen that causes many of the symptoms among IBD patients. These observations lead to the conclusion that the reconstruction of the gut flora, via the use of antibiotics, tremendously improves the condition of the mouse suffering with DSS induced colitis.

The antibiotics' annihilation of bacteria variability can be seen in the DGGE and bacterial analysis in Figure 7. Compared to the TD and PD mice given only water (seen in

Figure 4), the TD/AB and PD/AB mice has significantly less bands in the DGGE, which translates to less diversity in gut flora. This is further seen in the bacterial sequence analysis, as the PD/AB mice's colon is comprised of one single type of bacteria, as opposed to the TD mice's colon, which has three types of bacteria, yet mostly made-up of the same type of bacterium as the PD mice.

Under the antibiotic regimen, the *Clostridium* genera was completely destroyed, as well as the severe symptoms expressed by the mice without the antibiotics. As stated previously, many people suffering from IBD also become infected with *Clostridium deficile*. As stated previously, *C.deficile* causes severe irritation and damage to the intestinal lining when releasing its two types of toxin.

The huge improvements that the antibiotics provided (while keeping the diet consistent) also suggests many problems derived from IBD are influenced by intestinal flora. Had the immune system been as large of a factor as proposed pre-experiment, the antibiotics would not have had as much of an impact in the change of body weight and the maintenance of colon in the form of the colon length. However, the slight differences between the PD/AB and TD/AB mice cannot be overlooked. These weight changes could be accounted for by composition differences in the diet (again, most probably lactose derived), or how this nutrition influenced the mice bodies' immune system.

Conclusion

The use of antibiotics largely alleviated the symptoms that were present in their absence. This finding was independent of diets, that were comprised of slightly different components (mostly carbohydrate). This suggests that IBD patients can and should use therapeutic treatments that restructure their body's microflora. Reintroducing and reconfiguring one's gut microflora proved to dramatically improve symptoms, especially with the elimination of the *Clostridium* bacteria species. Similarly, patients can improve symptoms by avoiding lactose, as it is seen to induce negative consequences, seen in the preliminary experiment. Unfortunately, this experiment only allows us to speculate about what exactly the antibiotics did: did we destroy the pathogenic bacteria, or did we promote the beneficial ones? Future experiments to find exactly what bacteria causes what in the mice is a good start to find this answer. However, as there is a spectrum of causes and effects in science, the immune system cannot be completely overlooked as a potential factor in IBD symptomatic expression.

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⊗ Bachelors of Science: Veterinary and Biomedical Sciences ⊗ Graduated with HONORS (Schreyer's Honors College) ⊗ Every semester (8) earned Dean's List . Minor in Chinese

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Related Experience:

Ebensberg Animal Hospital *Ebensburg, PA* 130 hours volunteered

Town and Country Animal Hospital *Warriors March, PA* 150 hours volunteered

-Held Animals for blood draws, X-rays, administering anesthesia, nail clippings, and euthanizations.
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-Shadowed seven different Veterinarians, watching the intricacies and processes of surgeries, teeth cleans, and tattooings
-Worked with veterinarians on large animal farm calls, including calvings and pregnancy checks.

University of Pennsylvania Vet Program *Philadelphia, PA* 40 hours volunteered

-Learned the Ins and Outs of Veterinary School
-Worked four hour rotations in a number of specialty fields
-Held animals and better understood the anatomy of small animals

Job Experiences:

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University Park, PA

- Conduced my own research and discovered certain relationships between diet and DSS colitis.
- Handled and fed mice, as well as humanely carried out laboratory procedures.

Cosi Inc. - Barista and Trainer *Spring 2005 - Summer 2010*
Elkins Park, PA

- Helped people learn the ins and outs of a new company as a trainer.
- Studied many subjects that required many hours of learning and processing

Mitchell & Ness Inc. *Summer 2004*
Philadelphia, PA

- Moved up the ranks at Cosi Inc., having the opportunity to become a manager at age eighteen.
- Worked as a cashier at the Cosi restaurant.
- Helped escort customers to correct places at the different animal hospitals.