THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

Department of Veterinary and Biomedical Sciences

GUT MICROFLORA DISPLAY THEIR HANDIWORK: DIETARY IMPLICATIONS ON INTESTINAL BOWEL DISORDER

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SPRING 2012

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Immunology and Infectious Disease
with honors in Immunology and Infectious Disease

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Abstract:
Ulcerative Colitis, an intestinal bowel disorder, is affected by a host of external factors that impact the delicate immune system and gut microflora balances. Dietary components have been shown to both alleviate and exacerbate disease symptoms throughout the course of colitis pathogenesis. This study analyzes the role of two dietary components (lactose and fiber) in colitis progression using the dextran sulfate sodium (DSS) mouse model. A variety of diets and mouse subtypes were utilized in order to objectively identify the impacts of lactose and fiber. By obtaining colon/cecum lengths, analyzing their fecal DNA using denatured gradient gel electrophoresis (DGGE), and determining blood scores; the relative impacts of the nutrients on both the gut microflora composition and the mouse colitis symptoms were determined. Lactose had an overall detrimental effect on the colitis-induced mice as determined by their significantly reduced colon length and body weights whereas fiber produced mixed results. By examining the interplay between the gut microflora and immune system using these dietary factors, the role of our external surroundings in autoimmune proliferation begins to be revealed.

Table of Contents:

Introduction:

Dietary components, namely lactose and fiber, alter the disease progression and resulting symptom severity in mice induced with Dextran sulfate sodium (DSS)-colitis. Ulcerative colitis, one of two primary types of inflammatory bowel disease (IBD), is characterized by various demographic factors and an incompletely understood disease pathogenesis. Ulcerative colitis describes an inflammatory disease which is localized within the innermost lining of the large intestine (colon) and rectum, the disease isolation allows it to be distinguished from its fellow IBD, Crohn's disease (characterized by inflammation all along the digestive tract). The intestinal bowel diseases are predominately found in the developed world with UC currently afflicting between 500,000 and 700,000 individuals in the United States[1]. This observation is linked to the hygiene hypothesis which claims that increased levels of sanitation within developed countries decreases children's level of exposure to various infectious agents and results in depressed immune system development and corresponding autoimmunity. Ulcerative colitis rates are also increased in those with a family history of UC or with an Ashkenazi Jewish background; however, the disease seems to affect both men and women equally[2]. Many studies have shown a correlation between reduced sunlight exposure at higher latitudes and increased rates of IBD, postulating it to be connected to Vitamin D levels[3]. While the causes and therefore prevention methods of UC have yet to be discovered, stress and certain foods can trigger symptoms and flare-ups including abdominal pain, blood/pus in stools, diarrhea, fever and weight loss. A cocktail of drugs, anti-inflammatories, immune system suppressor, and antibiotics, operate to reduce inflammation, boost colon healing, and potentially lead to long-term disease remission. While both anti-inflammatories (corticosteroids and sulfasalazine) and immune system suppressors (cyclosporine and Remicade) diminish inflammation, anti-inflammatories decreases the activity of inflammatory pathway intermediates whereas immune system suppressors focus their action directly on down regulating the over-reactive immune system[2]. When treatment fails to curb disease progression, surgical colon removal provides a respectable alternative.

Immune Regulation: A Balancing Act

While the cause of Ulcerative Colitis remains largely unknown, the disease likely stems from the dysfunctional interaction between bacterial microflora of the gut and the mucosal immune system. Either the mucosal immune system is having an excessive immunological response to commensal bacterial gut species or dramatic alterations in gut microflora composition are eliciting pathogenic responses from a normal mucosal immune system[4]. An overactive mucosal immune response may be the result of defective T-cell populations, which attack commensal gut flora or T-reg cells (thereby failing to regulate gut inflammation). Recent studies have shown that two notable receptor domains on innate immune cells, TLR-4 (Toll-like receptor 4) and NOD-1 (nucleotide binding and oligomerization domain receptor), may play a role in priming the immune system for ulcerative colitis through increased T-cell activation and susceptibility respectively. Both structures act as pattern recognition receptors (PRR's) responsible for recognizing bacterial pattern-associated molecular patterns (PAMPs). TLR-4 binds LPS from incoming microorganisms and initiates an immune response through the development of T-helper cells whereas NOD-1 contains intracellular bacterial sensor proteins capable of initiating the innate immune response. Research shows that the IL-10 deficient mice (lacking a TLR-4 response) failed to develop colitis[5]. A recent genotypic TLR-4 analysis was completed in 98 ulcerative colitis patients and 145 controls, which identified the exact single nucleotide polymorphisms in TLR-4 associated with increased IBD susceptibility[6]. Two mutations (Thr399Ile and Asp299Gly) were found in the UC patients a statistically significant number of times more than controls and display the importance of the innate immune response in autoimmunity[6]. In the study of NOD-1 on colitis manifestation, three specific single amino acid changes within NOD-2 have been connected with increased ulcerative colitis prevalence in humans[4]. It should be noted that the complete elimination of either of these receptors (TLR-4 or NOD) is associated with an increased pathogenic bacterial burden as they are both PRR's essential for recognizing bacterial PAMPs and activating the immune response. These receptor domains represent just a small sampling of the possible

immune systems alterations that may be responsible for generating an abnormal mucosal immune response that recognizes its commensal bacteria as non-self.

While an abnormal mucosal immune response may provide the explanation behind UC pathogenesis, recent hypothesis's charge an irregular microflora composition as responsible for the autoimmunity. Trillions of commensal bacteria cells maintain a symbiotic relationship with gut; however, under certain conditions (immunodeficiency, relocation, etc.) they are capable of becoming pathogenic. Although inflammatory bowel disease has not been linked to a specific pathogenic organism, it has been found that IBD patients have higher levels of mucosa-associated bacteria then controls[4]. Our bacteria microflora sustain a low-level of inflammation with the gut; however, if bacterial intensity became too high it could drive this equilibrium out of balance. The predominant commensal bacterial species within the gut include but are not limited to *Bacteroides, Bifidobacterium, Escherichia, Clostridium*, and other anaerobes. The main organisms that evoke immune responses are toxigenic or invasive to gut epithelium. Generally, they exhibit unique properties such as increased gut epithelium adhesion by *E. coli* and immune system elusion by cell wall deficient organism such as *Enterococcus faecais* may play a role in disease progression[8]. While the commensal species do not usually parasitize the gut like the previously mentioned pathogens, they may be capable of it under certain conditions. Within the immune system, the main action in which commensal bacteria serve us with is preventing pathogenic bacterial establishment by occupying their potential niches. It has been observed that certain bacteria namely *Lactobacilli* and *Bifidobacteria* exhibit additional protective effects and that the level of these bacteria are lower in those with IBD[8]. An understanding of ulcerative colitis and other IBD's likely lies in the interplay between the mucosal immune response and the bacterial commensal species composition; however, continued research will further elucidate the required balance for immune regulation.

The Animal Colitis Model

After considering the potential explanations behind the causes of IBD, we will now turn our attention to the molecular pathways by which DSS (dextran sulfate

sodium) causes colitis-like disease in our mice. DSS is capable of causing both acute and chronic colitis; however, our studies took advantage of the DSS acute colitis model. Dextran sulfate sodium is toxic to basal crypt cells which, are found in the valleys between villi of the intestines and are essential in the production of stem cells needed to refurbish the epithelium of the crypt and villi[9]. Degradation of these cells leads to focal crypt lesions and secondary mucosal/ submucosal inflammation extending into both the colon and the cecum. High enough DSS concentrations and long enough administration times can lead to complete crypt dropout requiring healthy crypts to extend over large defective zones in order to regenerate the epithelium[9]. This accounts for the duration of time needed for the colon and cecum to heal in the colitis mouse model.

DSS induces acute colitis in mice by stimulating the innate immune response followed by the preliminary stages of a Th1-like response. One method of potential DSS pathogenesis involves its depletion of cellular tight junctions and the subsequent increase in gut permeability within the colon[10]. Heightened gut permeability allows increased levels of pathogens to both cross the gut epithelia and elicit the innate immune response. Mast cells respond to the initial disruption by releasing histamines and vasodilating the surrounding capillaries. The amplified vascular permeability allows phagocytes namely dendritic cells (DC), macrophages, and neutrophils to diffuse to the scene of infection, phagocytosis the trespassing pathogens, and release cytokines to prompt more phagocytes to the infection site. As previously discussed, PRR mutations (NOD2 located on the surface of dendritic cells) and the subsequent proinflammatory cytokine release (mainly IL-12 and TNF-alpha) facilitate neutrophilic diapedesis and the ulceration characteristic of colitis[11]. Over stimulation of this pro-inflammatory response leads to the superficial ulcers, goblet cell loss, and crypt distortion established in acute DSS colitis[10]. While acute DSS colitis predominantly initiates the non-specific inflammatory response, its influences are not confined to the innate immune response. The acute DSS colitis model has residual effects that alter the cell proliferation and response of the more specific adaptive immune system.

Nutrient Implications on the Gut

With an understanding of the intricate balance between the immune response and the commensal gut microflora as well as a basic understanding of the molecular level disease development caused by DSS, we examine the two variables of interest namely lactose and fiber. Many sources place both lactose and fiber on the "not recommended" dietary list for ulcerative colitis and Crohn's disease suffers; however, little research exists defending these allegations.

Lactose, a disaccharide sugar composed of glucose and galactose, is primarily found in dairy products including milk, cheese, yogurt, and ice cream. Much the same as those with a lactose intolerance, IBD patients with a lactose intolerance experience bloating, intestinal pain, and diarrhea. The intolerance stems from a lactase deficiency (the enzyme responsible for breaking lactose into it constitutive parts) and results in lactose build up in the upper small intestine[2]. The undigested lactose passes into the large intestines where it is fermented by a variety of bacterium producing carbon dioxide and hydrogen gas (causes bloating and fluctuation)[2]. The mixture of fermentation products increases the pressure in the large intestine, drawing water out of the body, and initiating diarrhea. While between 10 and 20% of IBD patients have sensitivity to dairy, the prevalence of lactose malabsorption in ulcerative colitis patients is not statistically different than the normal population[14]. However, there is a genetic link associated with lactase deficiency with Asians/ Native Americans far more likely to develop a lactose intolerance than North/Western Europeans. Lactose, along with many other nutrients consumed on a regular basis, create a unique set of microflora in the gut that may have disease potential under certain conditions. For instance, *Bifidobacteria* bacterium levels were significantly increased when excess lactose was available in the gut[15]. The *Bifidobacteria* seem to utilize the exogenous malabsorbed carbohydrates as a substrate in order to maintain their bacterial population more often than other bacterial species, which regularly colonize the gut[15]. The concentrations of the *Enterococci, Lactobacilli, and Staphylococci* also increased with lactose supplementation.

Fiber, the second dietary component of interest, enters the body through a number of dietary sources including fruit, vegetables, and whole grains. The nutrient materializes in one of two forms, either soluble fibers (attract water and slow the digestive process) or insoluble fibers (add bulk to the diet thereby preventing constipation). Our experiments employ insoluble fiber, which, is added to the mouse diets as alphacel (powdered cellulose). Insoluble fibers, in a sense, scrape the epithelial lining of the inflamed mucosa occasionally causing additional swelling and exacerbation to any ulcers or fissures. Cellulose executes the role of "dietary roughage" particularly well in mammals because most of them (mice and humans included) lack the necessary enzymes to lyse cellulose's beta-1, 4- glycosidic linkages. Studies support that high vegetable, fruit, and fiber intact can decrease one's risk of contracting either ulcerative colitis or Crohn's disease[16]; however, the effects of fiber on those who have already acquired the disease is less clear. One investigation used germinated barely foodstuff (GBF) as its source of dietary fiber and found it to have it effectively suppressed the DSS-induced colitis in their mice[17]. These results were based on both a visible rise in the activity level of the mice as well as increased SCFA (short-chain fatty acids) levels in their gut lumen[17]. SCFA are produced as colonic bacteria breakdown unabsorbed starch and polysaccharides. They have been shown to decrease the production of inflammatory mediators, repair damaged colon cells, maintain the microbial balance, and even prevent dysbiosis[18]. By reducing the number of TNF-alpha and nitric oxide molecules produced by the phagocytes, fewer molecules responsible for inflammation become stimulated. The short-chain fatty acids may be part of the justification for why IBD levels are higher in westernized countries; our diets are generally lower in dietary fiber and therefore lower levels of the SCFA's are produced to have their anti-inflammatory effects. While these results show fiber related to gut inflammation in a positive light, most medical professionals and IBD information sources recommend diets low in fiber to their patients. The various modifications fiber has on the gut are still being woven into an unambiguous overall picture; however, more research needs to be done to accomplish this.

The dietary implications of fiber and lactose on gut inflammation induced by DSS-colitis was our primary focus; however, we are also interested in the overarching role of Vitamin D in these complex relationships. Vitamin D is a lipid-soluble vitamin involved in bone growth and inflammation reduction. Vitamin D acquisition occurs either through absorption of sunlight and a subsequent photolysis reaction in the skin or through vitamin D rich foods (fatty fish and many supplemented dairy products). As previously mentioned, the hygiene hypothesis could provide some basis for increased levels of autoimmunity in developed countries but recent upsurge in vitamin D research demonstrates decreased levels of outdoor activity and low levels of vitamin D in the diet could have synergistic influences. IBD prevalence and vitamin D exposure have also established a geographic relationship as a proportionally higher number of IBD cases are found at higher latitudes, which have lower levels of environmental vitamin D exposure when compared to the equator. Human epidemiological studies illustrate IBD patients tend to have vitamin D deficiencies[19] and experimental models, which demonstrate that induced vitamin D receptor (VDR) deficiencies aggravate disease symptoms, further defend these findings[20]. While Vitamin D supplementation plays a protective role against intestinal bowel disease pathogenesis, the negligible amount of the vitamin used in our general experimental model likely had little impact on the overall conclusions.

Our studies seeked to understand both how dietary alterations affect the microbiota composition and how the microbiota species function within the dynamic network of gut immune regulation.

Materials/Methods:

Wild-type (WT) mice were administered DSS in their water source, monitored, and sacrificed in order to run a number of tests to look for variation in their bacterial flora content, immune system markers, and overall well-being. The six diets utilized to test the effects of the various dietary components on colitis pathogenesis included Harlan Teklad diet (Harlan company: Madison, Wisconsin #96348), chow diet (LabDiet: Quakertown, PA #5001), purified diet (State College, PA), purified high fiber diet (State College, PA), purified high lactose diet (State College, PA), and purified high lactose/high fiber combination diet (State College, PA). A listing of their respective constituents can be viewed in *Table 1.*

Purified Diet (g/kg)	**High Lactose Purified (g/kg)**	**High Lactose/ High Fiber (g/kg)**	**Teklad Diet (g/kg)**
Sucrose 218.3g Cerelose 440g Vitamin-free Casein 180g Arginine 2.4g Methionine 1.6g Glycine 4.5g Calcium Carbonate 21.8g Potassium Hydrogen Phosphate 4g Dipotassium Phosphate 5g Disodium Hydrogen Phosphate 12g Celluflour (Alphacel) 30g Choline Chloride 4g Sodium Ascorbate 1g Wesson Oil 50g Salt Mix 24g Water Soluble Mix 2g	Sucrose 218.3g Cerelose 240g Lactose 200g Vitamin-free Casein 180g Arginine 2.4g Methionine 1.6g Glycine 4.5g Calcium Carbonate 21.8g Potassium Hydrogen Phosphate 4g Dipotassium Phosphate 5g Disodium Hydrogen Phosphate 12g Celluflour (Alphacel) 30g Choline Chloride 4g Sodium Ascorbate 1g Wesson Oil 50g Salt Mix 24g Water Soluble Mix 2g	Sucrose 264g Cerelose 240g Lactose 200g Vitamin-free Casein 192g Arginine 2.4g Methionine 1.6g Glycine 4.5g Calcium Carbonate 21.8g Potassium Hydrogen Phosphate 4g Dipotassium Phosphate 5g Disodium Hydrogen Phosphate 12g Celluflour (Alphacel) 50g Choline Chloride 4g Sodium Ascorbate 1g Wesson Oil 50g Salt Mix 24g Water Soluble Mix 2g	Sucrose 264.8g Lactose 200g Corn Starch 150g Vitamin-free Casein 192g Methionine 3g Vitamin Mix 10g Calcium Carbonate 14g Cellulose 50g Corn Oil 50g Mineral Mix 35g Calcium Phosphate (dibasic) 31.2g

Table 1: Diet Comparison

DSS-induced colitis experimental model:

The WT mice were divided into experimental cohorts based on the number of mice available and their demographic characteristics (namely age and gender). The mice were matched based on their age/sex and group sizes were held relatively equivalent. The specific diets (chow, Teklad, purified, high lactose, high fiber, or high lactose/high fiber) were administered for two weeks with weekly weight monitoring. Feces samples were collected for denatured gradient gel electrophoresis (DGGE) analysis on Day 0. On day 0, the mice also began their 2.5% or 3.5% DSS treatment for a five day period. During the

five-day DSS treatment, the mice were weighed daily and sacrificed if they either lost 25% of their body weight from Day 0 or if they displayed life-threatening debilitating symptoms. The mice were then placed on regular water for a nine-day recovery period during which time they were weighed daily and monitored for significant weight loss. On day 14 of the experiment, feces samples were again acquired for DGGE analysis and the mice were sacrificed for further testing. The length of both the colon and the colon/cecum combined were measured and a corresponding blood score of the partially recovered organ was recorded. Small samples of the colon and cecum contents were attained for DNA analysis. Also, part of the distal colon was taken for histopathology and an RNA sample.

Analytical Techniques:

- Denatured Gradient Gel Electrophoresis:

DGGE is a molecular typing method used to distinguish between bacterial species based on their PCR-amplified DNA products. Normal electrophoresis differentiates between species based purely on the band size of the resulting PCR-amplified product; however, many gut bacterial species of interest have similar sized band and thus require a higher level of differentiation. The DGGE agarose gel contains an increasing gradient of denaturant and is therefore able to denature those bacterial DNA with weaker structures (higher proportion of A-T bonds compared with G-C) earlier during the process. The technique utilizes bacterial genotypic information in order to generate a specific band pattern for each unique bacterial population within the gut. The banding patterns are uploaded into databases where the resulting composite bacterial population can be compared to bacterial populations operating under slightly modified conditions (dietary nutrient alterations, knock-outs, vitamin-D level, etc.). Collecting feces both before and after DSS treatment allows the bacterial flora composition before and after treatment to be compared. The technique elucidates the role of the normal flora in the IBD immune response/ gut microbe balance.

- Cecum/ Colon Squeeze:

The cecum connects the ileum (the lower section of the small intestine) to the ascending colon as seen in *Figure 1*. This section of the digestive tract effectively reabsorbs water and salts from the waste products received from the small intestines and excretes mucus into the remaining waste products to ease its travels through the colon. The waste products (feces) which, traverse the colon leaving the body through the anus, are composed of large proportions of water, dead bacteria that were once involved in digestion, and indigestible fibers like cellulose. The stools also contain smaller proportions of cholesterol, inorganic salts, and mucus. Once the colon and cecum were effectively removed from the sacrificed mouse, they were cut open and small samples of their contents were acquired for bacterial species comparison. Often times if the mouse was found dead before it predetermined sacrifice date, these samples were unable to be collected.

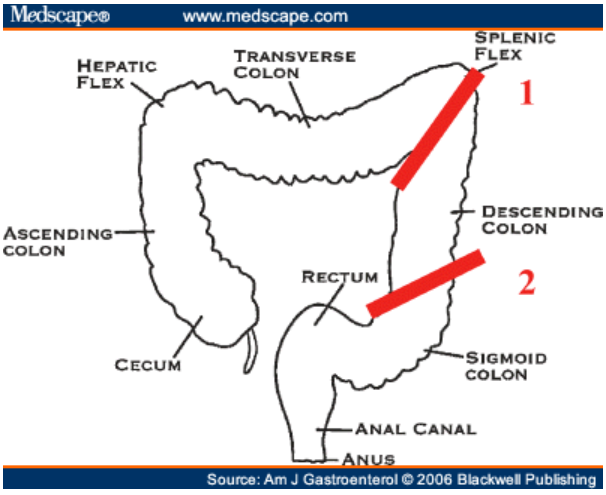


Figure 1: Colon Fractions

- Blood Score:

The blood score is a measure of the amount of blood found in the colon of the diseased mouse. The blood score ranges from 0 to 3 with zero being no blood found in the intestine and three being the colon is completely full of blood. While blood score determination is a slightly more subjective form of analysis, the results correlate well with the overall colitis severity in the diseased mice.

- Histopathology:

Post- colon/cecum extraction, a small segment of the mouse's distal colon was extracted for histopathology analysis. *Figure 1* illustrates the precise extraction points found in the descending colon, which was essential to ensure a certain level

of consistency between the sections of organ under microscopic examination. Histopathology analyzes tissues down to the cellular level and allows us to specifically compare the cellular structure of a diseased and non-diseased organisms colon. A healthy colon contains narrow, straight, unbranched crypt cells that have a relatively constant diameter. The surface epithelium of this healthy organ is characterized by a mixture of tall, columnar absorptive cells, endocrine, and goblet cells. Paneth cells are also commonly found in the cecum and proximal colon but can be found further down the distal colon during periods of chronic inflammation[21]. The lamina propria, a layer of loose, connective tissue found underneath the epithelium, holds a large number of chronic inflammatory cells and occasionally neutrophils. Inflammatory disease may be present if neutrophils relocate to the surface or crypt epithelium. Crypt bases that begin to separate from their thin smooth muscle (muscularis mucosae) due to lymphocyte build-up is also indicative of inflammatory disease[21]. The relative position and association between these components of the colonic lining are clarified by *Figure 2*. These results define the level of disease progression within the mice and determine whether the other phenotypic findings were due to the inflammatory disease or extenuating factors.

- Statistical analysis

The relative significance of the variations within the data was determined using an unpaired t-test. A p-value of less than 0.05 was deemed significant as they fell outside the 95% confidence interval and were likely not due to chance.

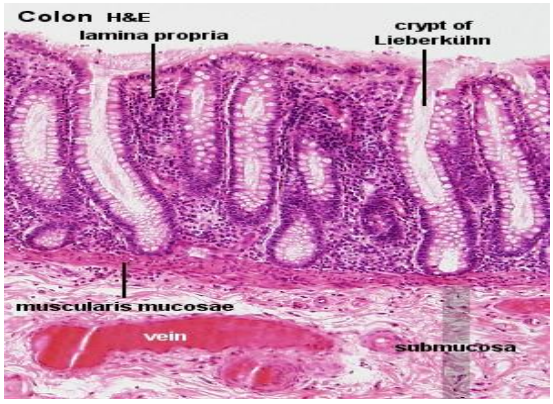


Figure 2: Cross-section of colonic lining

Results:

To investigate the role of lactose and fiber on colitis proliferation, we completed a number of dietary experiments on mice induced with DSS-colitis. The first set of experiments (*Figures 3-8*), employed to analyze the effects of dietary components on DSS-induced colitis, focused on the impact of high lactose on both VDRKO and RAGKO mice whereas the second set of experiments (*Figure 9-14*) studied the effects of both high lactose and high fiber on RAGKO mice.

The first set of experiments utilized two distinct forms of high lactose diet namely Teklad (a pellet rescue diet) and purifed high lactose along with their respective controls in Vitamin-D receptor (VDR) knock-out mice. While the mouse weights remained relatively stable during the five-day DSS administration period, the Teklad diet mice saw significant weight loss during the recovery period (*Figure 3*). During experimentation, two Teklad diet (high lactose) mice were mercy sacrified as they reached their 25% body weight loss threshold on day 8 and 11 respectively (*Figure 3*). The destructive effects of the Teklad diet on mouse gut epithelial cells can be more distinctively seen by analyzing the colon and cecum lengths of the sacrificed mice (*Figure 4*). Prior to DSS-treatment, the mice in all four treatment groups had similar body weights and colon lengths. After the fourteen-day treatment period, the chow mice had significantly longer colon lengths when compared with both the Teklad and purified high lactose groups. While the dry Teklad caused significant pathogenic colon shortening, the purified high lactose diet uncovered longer colon lengths when compared with the purified diet; however, these findings were not significant (*Figure 4*). Only the two lactose supplementation diets saw mouse weight loss greater than 5% (*Figure 3*). However, the disease severity contributed to the Teklad diet over the purified high lactose diet can be seen through both their substantial weight loss and their significantly smaller colon lengths (*Figure 3 and 4*).

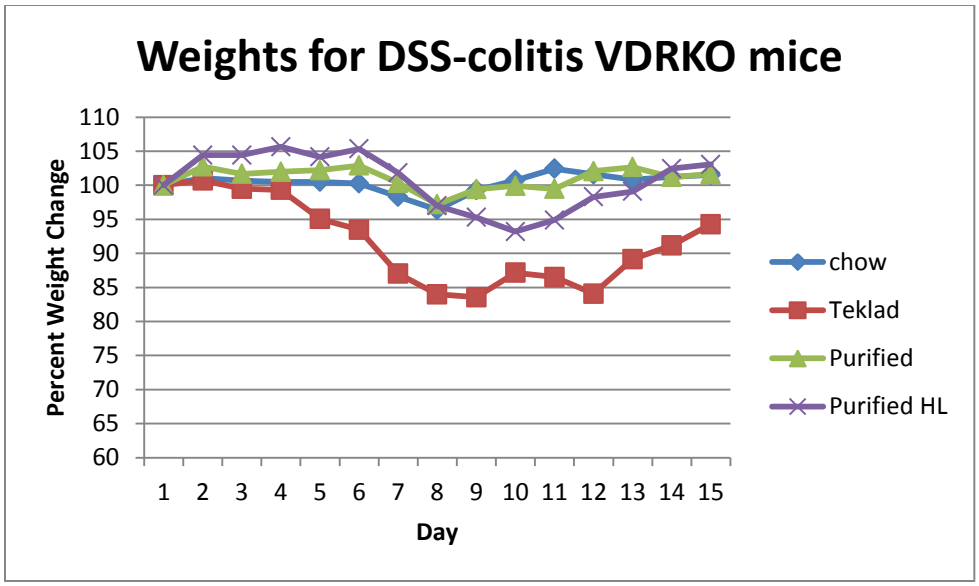


Figure 3: VDRKO DSS-induced Colitis Body Weights

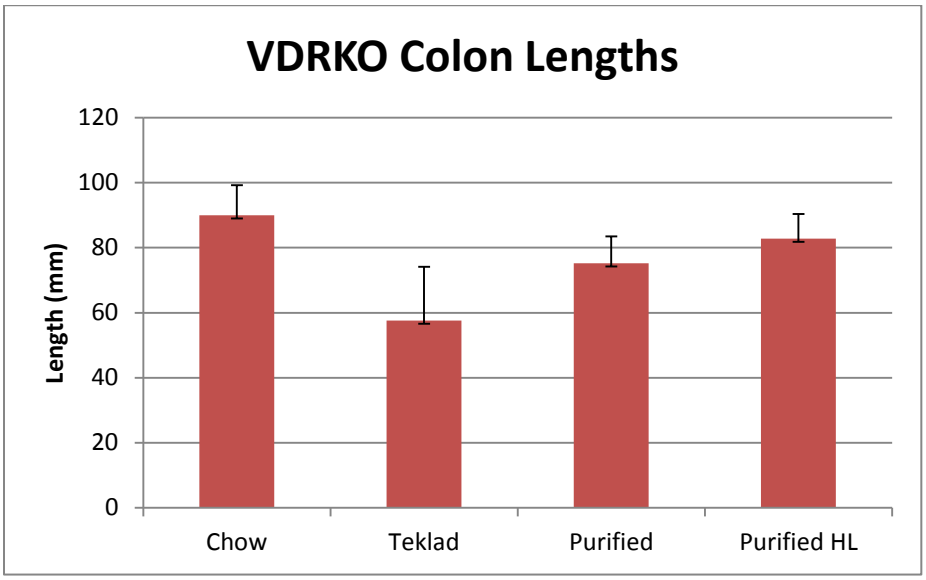
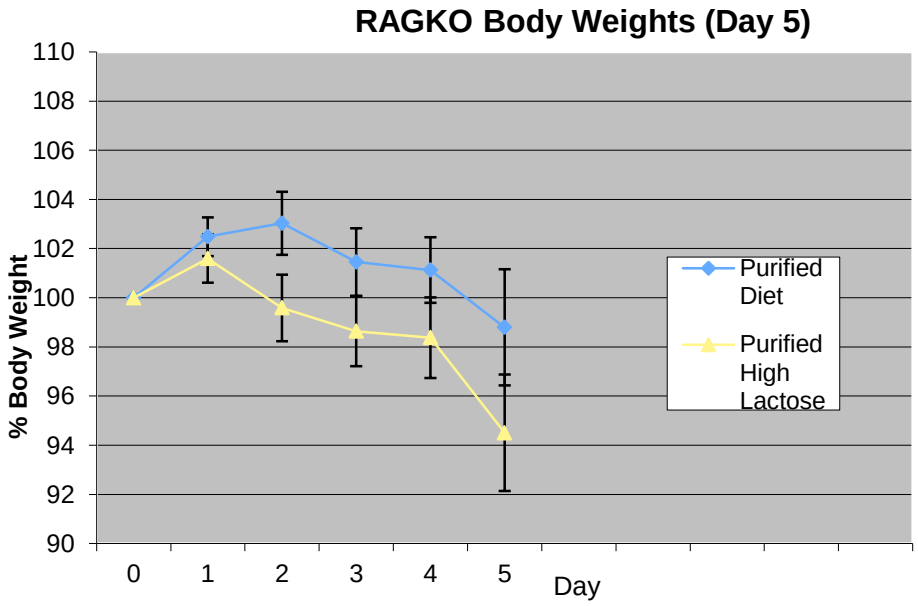


Figure 4: VDRKO Colitis Colon Lengths

A slightly modified experimental procedure was followed in Recombination Activating Gene knock-out (RAGKO) mice to determine how lactose impacts their altered immune system. The RAGKO high lactose diet experiment summarized by *Figures 5-8* used the same diets (only substituting purified high lactose/ high fiber for chow) and experimental conditions as the previous experiment completed in the VDRKO

mice. By repeating the experiment, the DSS colitis mouse diet experimental protocol was enhanced and the impacts of the absence of different immunity molecules were able to be compared. In the VDRKO experiment, all the mice were retained for the full 14 day experiment (with the exception of 2 high lactose pellet mice which, were mercy sacrificed on Day 8 and 11 respectively after falling below the 25% weight loss threshold). The subsequent RAGKO experimental protocol included two different points of analytical sacrifice: one after the five days of DSS treatment and another after the full 10 day treatment and recovery period. The body weight fluctuations throughout the experimental period are compiled into two separate displays based upon when the mice were sacrificed. The purified and purified high lactose treatment groups were each split into two subgroups based upon their day of sacrifice. *Figure 5* displays the weights changes for the purified and purified high lactose mice that were sacrificed after receiving five days of DSS treatment. Paralleling this diagram, *Figure 6* displays only the fluctuating weights of the mice that were destined for the full five-day DSS treatment and the five-day recovery period. There were ten mice in each experimental group (purified, purified high lactose, Teklad, and purified high lactose/ high fiber) with half of the purified and purified high lactose population being sacrificed midway through the experiment. There are no significant weight changes between the purified and purified high lactose diet mice after the five day treatment period or the full experimental course (*Figure 5*); however, greater disparities occur between these treatment groups and the Teklad diet mice as seen in *Figure 6*. The Teklad diet mice demonstrated the most severe weight loss and the high lactose/ high fiber diet displayed intermediary weight reduction that fell in between the purified and Teklad diet groups (*Figure 6*). The RAGKO purified high lactose mice displayed similar weight loss trends as the VDRKO mice, with the purified high lactose mice experiencing slightly greater weight loss than the purified mice (compare *Figure 4 and 6*). While the Teklad mice discernibly displayed the most significant weight loss, these result need to be handled with caution since data past day 6 was only available for one mouse due to mercy sacrifice (*Figure 6*). The colon lengths of the RAGKO mice follow a similar pattern to the VDRKO mice as the Teklad diet

mice have significantly shorter colon lengths when compared to the other three test groups and the purified high lactose mice have slightly longer lengths than their purified diet counterparts (*Figure 7b*). The colon seems to have elongated during recovery; however, the length discrepancies between day 5 and 10 are not statistically significant (*Figure 7a and 7b*). Just has the purified high lactose/ high fiber diet group has intermediary weight loss; they also have intermediary colon lengths (*Figure 7b*). Blood score (*Figure 8*) is yet another measure of symptom severity and the Teklad diet mice were the only class to develop a blood score (average of 1.9). Both the VDRKO and RAGKO DSS-induced colitis lactose diet experiment observe increased disease severity in the Teklad diet mice characterized by substantial weight loss and colon length reduction compared with mice receiving other dietary variations.



F*igure 5: RAGKO DSS-induced Colitis Weights (Day 5)*

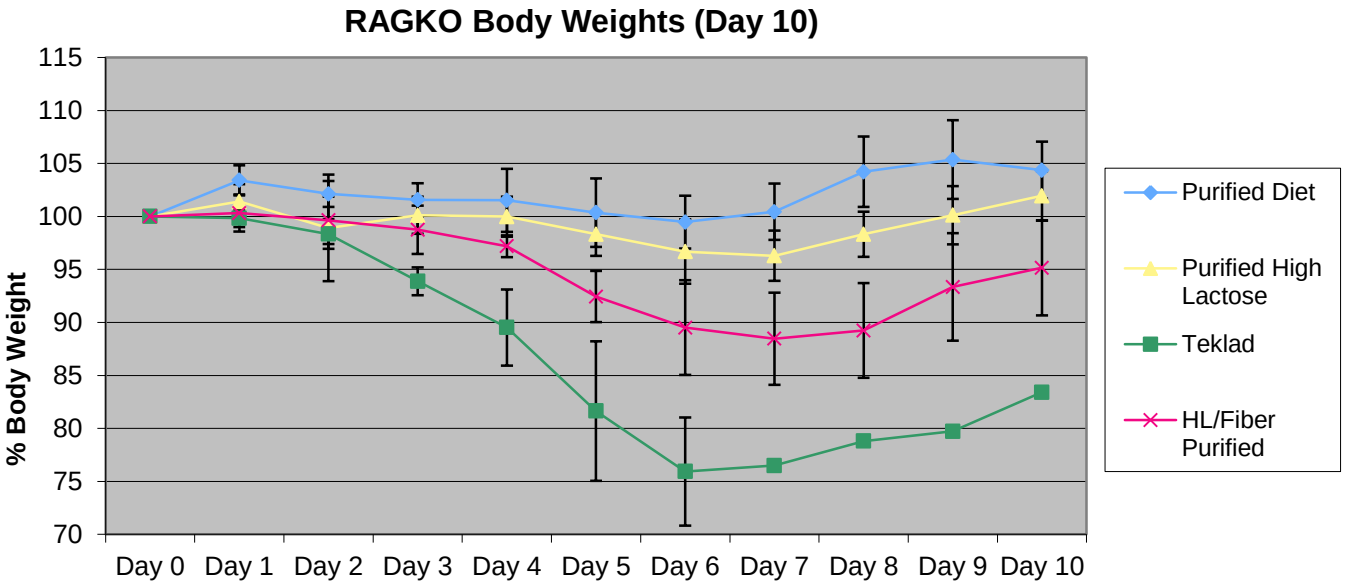


Figure 6: RAGKO DSS-induced Colitis Weights (Day 10)

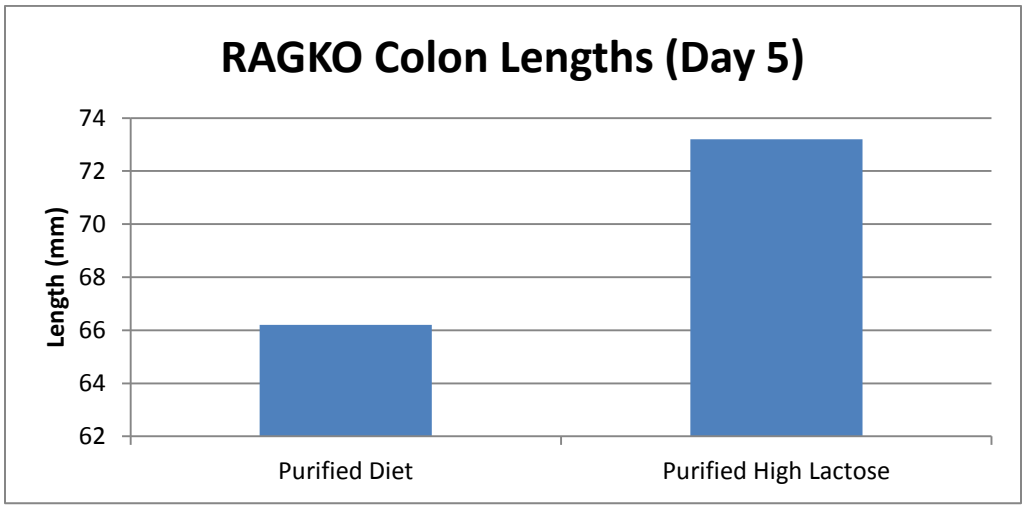


Figure 7a: RAGKO Colon Lengths sacrificed on Day 5

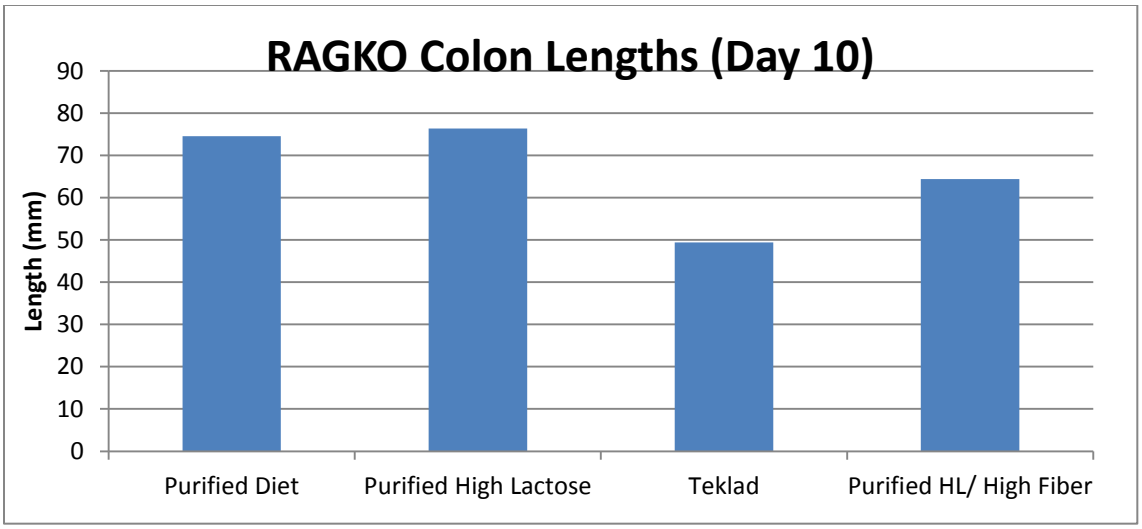


Figure 7b: RAGKO Colon Lengths sacrificed on Day 10

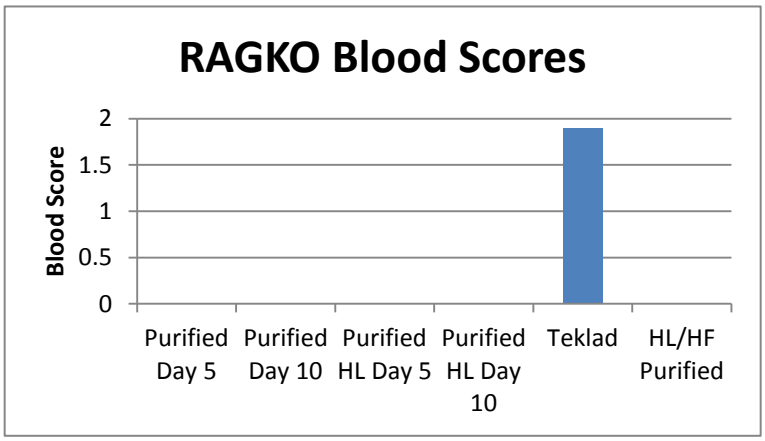


Figure 8: RAGKO Blood Scores

As another study of interest, the second branch of experiments, represented by *Figures 9-14,* examined the collective implications of high fiber and high lactose on RAGKO mice induced with DSS-colitis. The first experiment (*Figure 9-11*) induced colitis in 10 WT mice using 3.5% DSS in order to examine the difference in disease severity between the five mice on purified diet and the five mice on purified high lactose/high fiber diet. The treatment groups displayed no significant difference in their weight loss over the 14-day treatment and recovery period unlike the VDRKO and RAGKO mice in the previous experiments (*Figure 9*). The results also produced no significant difference

between the treatment groups colon lengths; however, when cecum length was added to the measurement, the mice in the high fiber/ high lactose group had significantly longer colon/cecum lengths than the purified diet mice (*Figure 10*). The WT mice provided an objective foundation to run denatured gel gradient electrophoresis (DGGE) in order to compare the relative gut flora of the various mouse dietary treatment groups. After isolating fecal DNA and amplifying with PCR, DGGE created distinct banding patterns which, correlated with the bacterial species present in the treated mouse's gut. DGGE does not always generate an accurate account of the species present within the gut and it is therefore often presented with a DNA sequence analysis. However, DGGE is capable of depicting distinct microbial communities as seen through the variable placement and thickness of bands between the control and high lactose/high fiber mice gels (*Figure 11*). While the groups have discrete banding patterns, the distinctions are not shared amongst all members of the treatment group making it difficult to gather any conclusive results from the figure. For comparisons sake, the DGGE gel comparing WT mice fed chow and Teklad diets can be viewed in *Figure 12*; however, this gel also contains inconsistencies between members of the study groups.

The high lactose/ high fiber experiment was repeated in RAGKO mice using 2.5% DSS and the same five-day treatment and nine-day recovery period was utilized. This experiment also added a purified high fiber treatment group to supplement the original purified high lactose/ high fiber and purified diet groups. The six high fiber/ high lactose and six high fiber mice saw a decrease in weight compared with the six purified diet mice; however, the weights were not significantly different (*Figure 13*). The high fiber/high lactose RAGKO mice experienced similar levels of weight loss (around 10%) (Figure 13) to the RAGKO purified high lactose/ high fiber tested in earlier experiments (*Figure 6*). It is also important to note that the mice given both the high lactose and high fiber saw a greater weight loss than the mice only given the high fiber supplemented diet (*Figure 13*). *Figure 14* displays little variation between the colon and cecum lengths of the three treatment groups. The second set of high lactose/ high fiber experiments

produced fewer statistically significant findings compared with the initial experiments which focused on lactose.

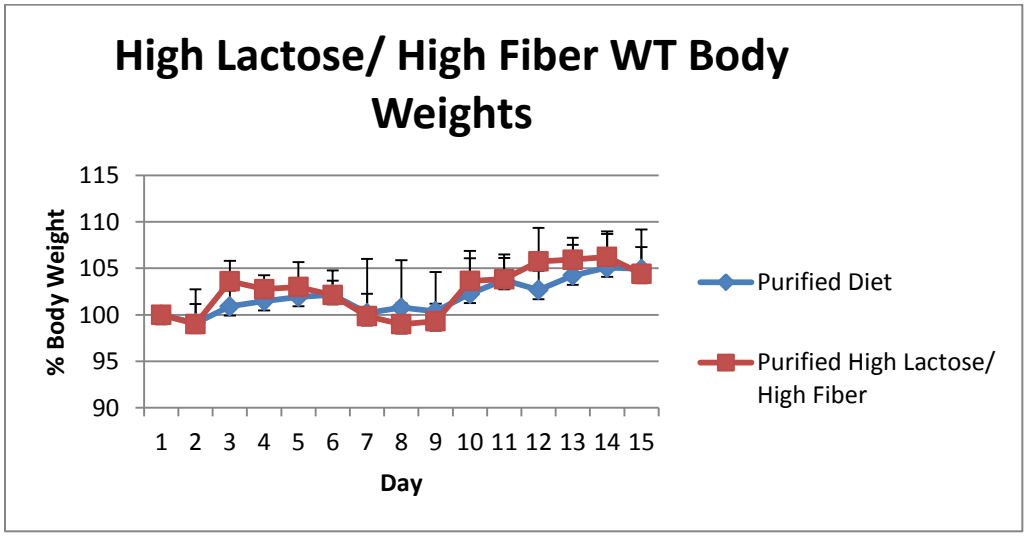
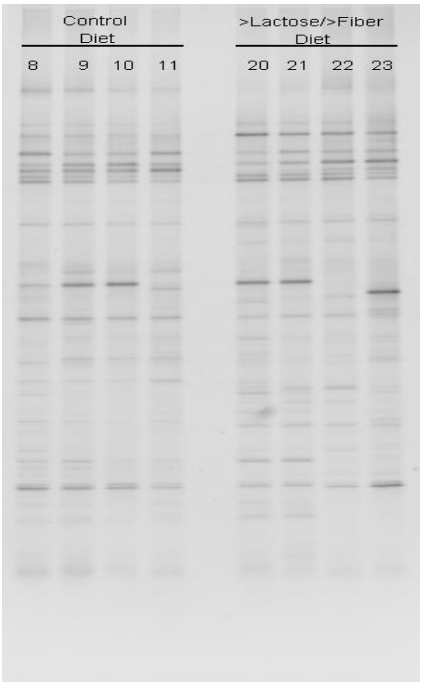


Figure 9: Purified High Lactose/ High Fiber WT Body Weights



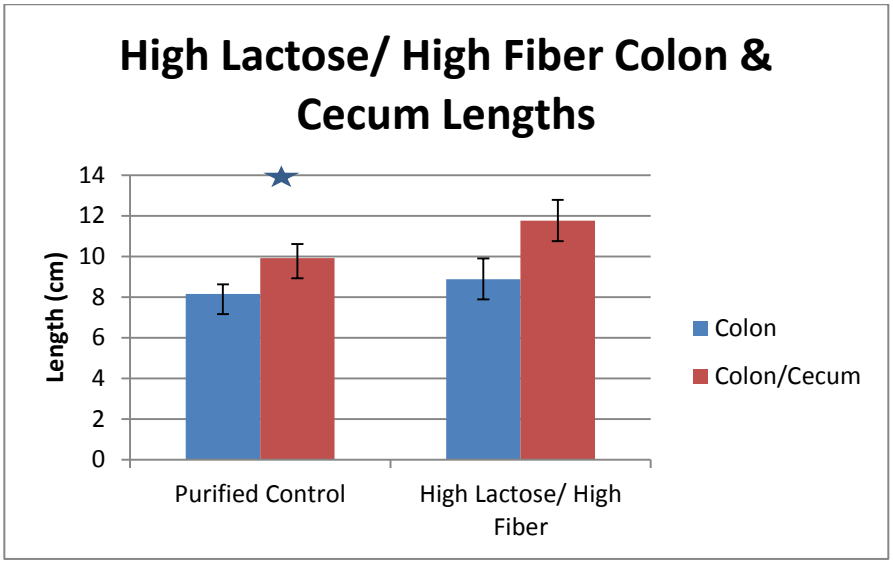


Figure 10: High Lactose/ High Fiber WT Colon Lengths

Figure 11: Relative Gut Flora in Wild-type mice (Purified vs. HL/HF)

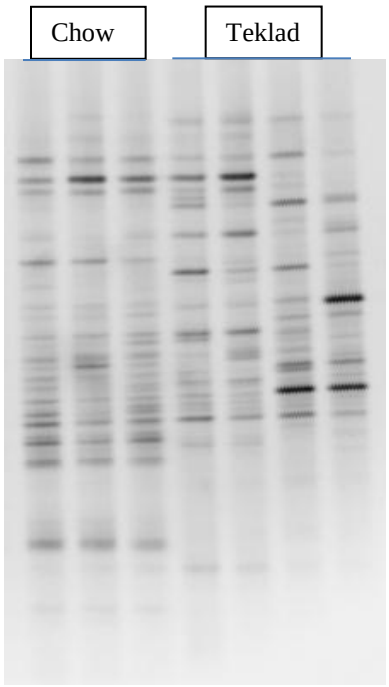


Figure 12: Relative Gut Flora in Wild-type mice (Chow vs. Teklad)

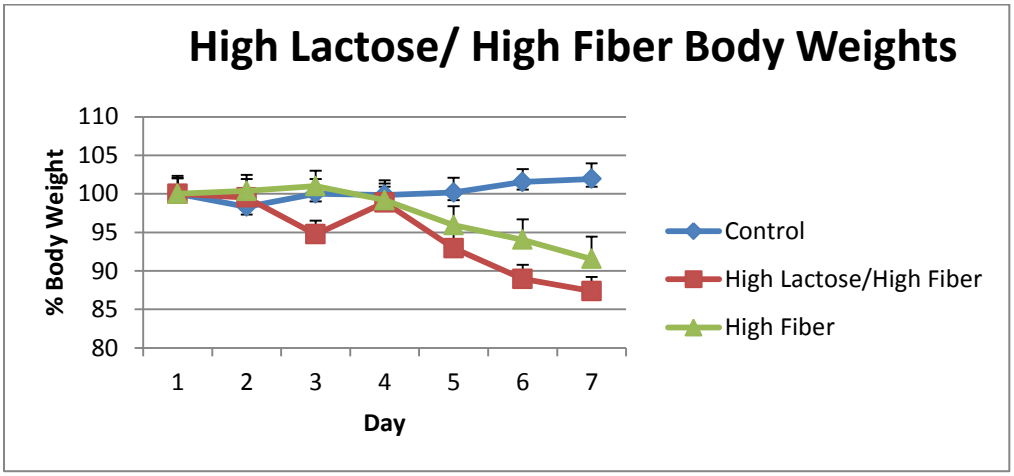


Figure 13: High Lactose/ High Fiber RAGKO Body Weights

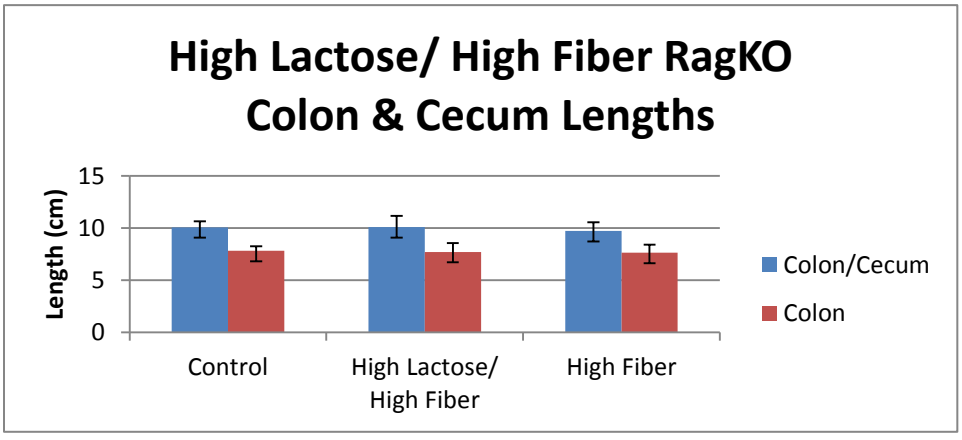


Figure 14: High Lactose/ High Fiber RAGKO Colon Lengths

The final section of the study was a thorough analysis of dietary components to determine whether or not the lactose and/or fiber were inherently responsible for the disease exacerbation. The purified, purified high lactose, purified high fiber, and purified high lactose/high fiber diets are all very similar with slight variations in their lactose and/or fiber levels based upon their names. The Teklad recovery diet uses cellulose as its source of fiber and corn starch as its form of glucose instead of dextrose. The dietary components are consistent throughout and our high lactose/ high fiber purified diet provides an accurate representation of the Teklad diet as demonstrated through *Table 1*.

Discussion:

Using different mouse strains, diets, and experimental protocols allowed us to wade through the myriad of confounding variables in order to procure real conclusions regarding the impacts of both lactose and fiber on colitis pathogenesis. The dietary components of interest were studied using two different experiments which produced two distinct sets of results. The first, which looked at the effects of high lactose (in purified and pellet form) on both VDRKO and RAGKO mice, showed significant implications on colitis disease severity in the mice treated with high lactose pellets (Teklad diet). The second set of experiments focused on the synergistic effects of fiber and lactose in both wild-type and RAGKO DSS-induced colitis mice and determined that while the high lactose/ high fiber diet did result in increased symptom severity the differentiation from the control group was not significant. While the ability of high lactose but not high lactose/ high lactose to produce significant disease severity does not intrinsically make sense, it is important to highlight that the high lactose pellets caused the significant results in the first set of experiments whereas purified high fiber/ high lactose diet was used in the second set of experiments.

The initial experiments determined that high lactose (Teklad diet) caused significant disease aggravation in both RAGKO and VDRKO mice. However, the RAGKO mice demonstrated more severe disease progression than the VDRKO mice as

quantitatively observed through their reduced weights, shortened colon/cecum, and notable blood scores. Vitamin D regulates the immune systems antimicrobial activity and balances the body's enteric bacteria[22]; therefore, by preventing Vitamin D uptake using VDRKO, mice become predisposed to colitis. On the other hand, RAGKOs eliminate an organism's entire adaptive immune response leading to more severe disease respectively. While the disease model primarily targets the innate immune response, the early stages of T-cell development are required to stave off late phase disease progression. The unavailability of these acquired immune cells caused significant weight loss late in the recovery phase (especially evident in the Teklad diet mice) (*Figure 6*).

Considering the experiments as a whole, a few other trends can be extracted. First, fiber as utilized in the high lactose/high fiber diet had negative ramifications on the RAGKO mice (*Figure 6 and 7b*) yet seemed to have a protective effect on the healthy WT mice (*Figure 10*). It may be possible that the gut bacterial imbalance in the immunocompromised mice gut cannot handle any sort of indigestible products whereas the colitis model in healthy mice doesn't disturb the gut flora composition as much and therefore causing less damage. Research detailing the signaling mechanisms in the gut revealed that dietary components can simultaneously have positive and negative effects on the gut[23]. Various dietary components are capable of both eliciting a secretory IgA antibody response and developing a state of systematic hyporesponsiveness (characterized by a reduced response to immune markers)[23]. There relative responses depend on the immune cells available (RAGKO cannot elicit an antibody response without B cells), the severity of their disease, and their acquired gut composition. Recent trials completed in healthy piglets revealed that Nutriose (a soluble fiber diet) had positive effects on both their immune regulation and gut composition[24]. Nutriose stimulated the production of butyrate which correlated with a shift in microbiota towards supportive butyrogenic species such as Fusobacterium, and Bifidobacterium[24]. While fiber may stimulate the growth of vital bacterial populations, its abrasiveness may cause exaggerated colitis symptoms (ulcer, fissures, abscesses, etc.) initially.

Excluding the previously discussed high lactose/ high fiber anomaly, a second uniting trend is the consistent results between the various diets regardless of the mouse strain (RAGKO, VDRKO or WT). The purified diet mice don't lose any substantial amount of weight (*Figure 6 and 9*), the Teklad diet always produces the most severe symptoms (*Figure 3, 4, 6, and 7b*), and purified diets fortified with lactose or fiber yield oscillating intermediary results. These parallels required a careful examination of the dietary make-up in order to pinpoint the relevant factors. The dietary comparison (*Table 1*) displays the full repertoire of components; however, the focus should be placed on the lactose, celluflour, and cerelose (dextrose) levels. Considering that cerelose (dextrose) is a completely hydrogenated form of cornstarch[25], there is little support that these carbohydrate constituents are the bulk of the issue. However, the incorporation of water into dextrose provides vital hydration for the diarrheic mice and may make the food more palatable. Observations reveal that the Teklad diet mice may lose the majority of their weight due to fluid loss through the combined effects of diarrhea and diminished fluid intake. Celluflour, a finely ground up form of cellulose, may provide slightly diminished symptom aggravation in the purified diet mice as compared with the Teklad diet mice due to its smaller particle size. However, fibers abrasiveness is also not likely the culprit behind these enhanced colitis symptoms. The evidence implicate lactose as predominately accountable for the heightened disease manifestation as demonstrated through both the severity of the teklad diet and the side-by-side comparison of the purified/purified high lactose diets. Recent studies revealed that a higher proportion of intestinal bowel disorder patients are considered to be lactose sensitive (characterized as a reduced ability to degrade lactose because of insufficient or transformed lactase enzymes) than previously thought[26]. Lactose accumulation escapes the small intestine and requires breakdown by colonic bacteria into short chain fatty acids (SCFA) which, decreases the pH of the large intestine. Shifts in the microbiota composition follow these pH alterations, causing increased levels of lactobacillus, bifidiobacterium and coliform reductions[27]. While lactobacillus and bifidiobacterium strains generally have protective effects against colitis[28], dramatic shifts in gut flora

composition due to faulty lactose breakdown have been linked to colitis flare-ups[29]. Furthermore, lactose not only modifies the composition of the gut but also alters bacteria's ability to attach and colonize the gut epithelia. Siayllactose, for instance, was shown to facilitate the attachment of both Escherichia coli and Vibrio cholera by acting as their energy source[30]. This form of lactose also demonstrated proinflammatory action in the DSS-induced colitis model which, may further link it to increased disease[30]. Our studies compiled with recent research provide the foundation needed to implicate lactose as colitis symptom instigator.

The shortening and eventual regeneration of the colon during DSS-treatment is another feature of the results that bares further examination. The colon has the ability to regenerates its inner layer on a weekly basis, a quality that is not lost even after severe epithelial disturbance. This regeneration is typified in the RAGKO experiment. The RAGKO high lactose study (*Figures 5-8*) incorporated an analytical sacrifice at day 5 of the 10 day experiment in order to get intermediary result on the weights and colon/cecum lengths. As expected, the colon lengths after recovery (Day 10) are significantly longer than those acquired directly after the DSS administration. The shortening of the colon is due to the hypertrophy of the muscularis mucosae (a thin layer of smooth muscle which lies basolateral to the mucosal epithelium) which, is induced by inflammation[31]. The diminished length or complete loss of crypts also contributes to the shortening of the large intestine as seen in *Figure 2*. During the recovery phase, the DSS-induced innate inflammatory response diminishes and the colon begins to elongate and regain its integrity. Healing zones, activated by growth factors, cause cells lining gastrointestinal glands to begin to secrete epidermal growth factor receptor (EGFR) in order to respond to epidermal growth factor (EGF)[32]. The growth factors stimulate crypt regeneration and the muscularis mucosa relaxes allowing the colon to return to its original length. While the cecum (a pouch connecting small intestine to large intestine) and colon are both affected by colitis, the cecum length did not show any substantial length changes (consistently between 20 and 25mm) whereas the colon length varied from 49-85mm throughout the various experiments. These

findings suggest that the majority of the inflammation and disease is localized in the transverse and descending colon. The inclusion of the colon/cecum length measurement decreases the level of error associated with the colon length. The dietary experimental evidence supports that the immune system has an innate ability to heal itself even after environmental disturbances.

These studies prove the progression of colitis is affected by the complex interplay between the gut flora, the immune system, and the diet. The increased levels of lactose coupled with the inability for mice to get adequate hydration through the Teklad diet led to the most severe colitic symptoms as exemplified through their weight loss and colon shortening. The debilitating effects of the high lactose Teklad diet on mice with DSS-induced colitis was repeated and reinforced by our own purified high lactose diet (only to less severe extent). Both the fiber and lactose altered the composition of the gut flora (as demonstrated in the DGGE analysis), causing the colon to struggle to regenerate after DSS exposure. While fiber occasionally demonstrates a protective effect, lactose inclusion within the diet frequently leads to early mercy sacrifice, severe bloody stools, and an incapacitated state.

The DSS-induced colitis model mimics human colitis pathogenesis allowing us to make connections between our research and the diseases development in humans. Our findings both contribute to appropriate dietary guidelines for those suffering from IBD and add to our understanding behind unknown cause and progression of IBD. As the data suggested, fiber has had protective effects as individuals with IBD experiences fewer flare-ups and hospitalization while consuming 15 grams or more of fiber per day[33]. The effects of lactose in human with colitis also parallels our studies as it has been proven to both increase epithelial permeability and cytokine production which, contributes to the gut inflammation associated with colitis[33]. The effects of the dietary alterations are tightly linked with shifts in the gut flora which is why probiotics are the way of the future. By continuing to unravel the vast complexity of the microbiome, its relationship with the immune response and eventually its ability to cause intestinal bowel disorders will be revealed.

Acknowledgements:

I would like to thank my fellow undergraduate lab mates (Tori Holden, Laura Rust, and Gabe Innes) for their collaborative efforts, my mentors (Jot Hui Ooi and Rhonda Smith) for their statistical assistance and advice, and my thesis advisor (Margherita Cantorna) for her patience and unyielding support.

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