

FLAXSEED AND BUCKWHEAT SUPPLEMENTED DIETS ALTERED
ENTEROBACTERIACEAE PREVALENCE IN THE CECUM AND FECES OF MICE

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ABSTRACT

Dietary intake may cause variable bacterial prevalence in the gastrointestinal tract. The objective of this research was to determine the prevalence of *Enterobacteriaceae* in the cecum and feces following flaxseed and buckwheat supplemented diets. Seventy-two C57BL/6J male mice were randomly assigned to a diet group and fed for eight weeks: high fat (45% Kcal fat); 10% whole flaxseed (45% Kcal fat); 6% defatted flaxseed (45% Kcal fat); 4% flaxseed oil (45% Kcal fat); 10% buckwheat (45% Kcal fat); and low fat (16% Kcal fat). Significant differences in the prevalence of *Enterobacteriaceae* in the cecum ($p < 0.0348$) and feces post treatment ($p < 0.0033$) were observed. The groups with the highest prevalence of *Enterobacteriaceae* were whole flaxseed, buckwheat, and defatted flaxseed. The groups with the lowest prevalence were flaxseed oil and high fat. Our results indicated that a positive relationship exists between high fermentable fiber diets and *Enterobacteriaceae* proliferation.

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LIST OF ABBREVIATIONS

BMI.....	body mass index
BRFSS.....	Behavioral Risk Factor Surveillance System
BW.....	buckwheat diet
CFU.....	colony forming unit
DF.....	defatted flaxseed diet
FO.....	flaxseed oil diet
g.....	grams
GI.....	gastrointestinal tract
HF.....	high fat diet
IACUC.....	Institutional Animal Care and Use Committee
Kcal.....	kilocalorie
LF.....	low fat diet
NHANES.....	National Health and Nutrition Examination Survey
NDSU.....	North Dakota State University
PBS.....	phosphate based saline
RT-qPCR.....	real time quantitative polymerase chain reaction
RS.....	resistant starch
SCFA.....	short chain fatty acids
SDG.....	secoisolariciresinol diglucoside
spp.....	species
WF.....	whole flaxseed diet

CHAPTER 1. INTRODUCTION

The United States is experiencing an obesity epidemic that is continuing to threaten the future health and wellness of our society and is exhausting our medical system (Centers for Disease Control and Prevention, 2014). Researchers continue to investigate a variety of theories to better understand the complex etiology of obesity in order to target and eliminate the risk factors and behaviors contributing to obesity. While the most commonly understood factors related to obesity are a sedentary lifestyle, excessive consumption of energy dense food, and genetic predisposition, other concepts as to the cause of obesity are still being explored (Harris et al., 2011).

One emerging theory for the cause of obesity suggests that some microorganisms, whether present or absent in the gastrointestinal (GI) tract, correlates with overweight and obese status. Researchers have observed that during weight loss, bacterial species that are considered beneficial in the body increase, while there is a decrease in detrimental species (Ley et al., 2005). Although a complete mechanism for this shift in microorganisms in obesity is not yet identified, there is evidence to support that the species of bacteria present in the colon correlate with weight changes and improvements in energy expenditure in obesity (Santacruz et al., 2009, 2010).

The GI tract is composed of a normal bacterial flora necessary for activities such as the fermentation of carbohydrates and short chain fatty acid production (Karlsson et al., 2012). While some species of bacteria such as *Lactobacillus* and *Bifidobacteria* are known for their roles in the prevention of infections and immunity, others belonging to *Enterobacteriaceae* including *Salmonella*, *Escherichia coli*, and *Shigella* are known for being detrimental when overgrown within the GI tract (Santacruz et al., 2010). Higher prevalence of *Enterobacteriaceae*

in the GI tract may be more prevalent in those who are obese compared to those who are normal weight.

Prebiotics are becoming a commonly used method to maintain the colon's microflora (Hijova, Chmelarova, & Bomba, 2009). Prebiotics assist with the growth of GI bacterial species that improve the health of the host. These types of bacteria enhance the immune response in the gut and alter the pH concentration of the colon. These activities inhibit the growth of harmful bacteria and encourage the growth of health-promoting bacterial populations.

Fermentable fibers exhibit a prebiotic function by stimulating the growth of health-promoting bacteria (Bertkova, et al., 2010). Fermentable fiber properties exist in a variety of natural fibrous food products. Two examples of plants with high fermentable fiber content are flaxseed and buckwheat, which indicates they have potential to function as a prebiotic (Kristensen et al., 2012). The supplementation of these into the diet could lead to an increase of beneficial bacteria and also a decrease in detrimental bacterial species (Skrabanja et al., 2001).

Statement of the Problem

Obesity is a highly complex and chronic medical condition that requires serious attention. While science does not completely understand the mechanism of obesity, it is important to continue efforts to determine what factors contribute to the presence or absence of the condition. One emerging concept within obesity research is the effect of microbial prevalence within the GI tract in relation to the occurrence of obesity. Researchers have observed a correlation between weight loss and a corresponding increase of beneficial bacteria as well as a decrease of detrimental strains of bacteria in the colon. An imbalance of microbial populations within the GI tract between those that are beneficial and those that are detrimental may increase the risk for obesity (Ley et al., 2005; Santacruz et al., 2009, 2010). The focus of this research was to

contribute to the conceptualization of the mechanism between bacterial species in the GI tract and their effect on weight maintenance and the development of obesity.

Purpose of the Study

The purpose of this research was to identify the potential variations in *Enterobacteriaceae* prevalence in the cecum and feces of mice when fed diets supplemented with flaxseed or buckwheat. *Enterobacteriaceae* are often considered detrimental species of bacteria when found in excess in the human colon. These particular species, however, are able to proliferate in the colon in low prevalence without causing immediate harm or distress such as vomiting or diarrhea (Janda & Abbott 2006). However, *Enterobacteriaceae* may also be related to other conditions, such as obesity. (Santacruz et al., 2010; Karlsson et al., 2012).

Our goal was to determine whether supplementation promotes the growth or reduction of *Enterobacteriaceae* in the colon and presence of *Enterobacteriaceae* shed in the feces. The potential variability of microbial species present in the GI tract of obesity model animals in relation to body weight, specifically caused by the supplementation of flaxseed and buckwheat in the diet was evaluated.

Hypotheses

The first hypothesis was that flaxseed and buckwheat supplementation would proliferate *Enterobacteriaceae* both in the cecum and shed in the feces. The second hypothesis was that we would observe increased weight gain among the high fat groups. However, the groups fed with fiber from flaxseed and buckwheat would have lower overall weight gain. The third hypothesis was that the groups with the most bacterial proliferation would be the groups with the lowest overall weight gain.

Definition of Key Terms

Buckwheat (Fagopyrum esculentum). A pseudocereal containing large amounts of protein, starch, and vitamins as well as antioxidant properties (Kim, Son, & Lee, 2012).

Cecum. The first part of the large intestine, forming a dilated pouch distal to the ileum and proximal to the colon (Mahan et al., 2012).

Dietary Fiber. Nondigestible (by human digestive enzymes) carbohydrates and lignin that are intact and intrinsic in plants (Gropper & Smith, 2012).

Fermentable Fiber. Stimulate the production of bacteria in the digestive tract and can also generate short chain fatty acids. It includes fructans, pectin, gums, psyllium, polydextrose, and resistant starch. Some cellulose and hemicellulose are also included (Gropper & Smith, 2012).

Flaxseed (Linum usitatissimum). An oilseed crop for industrial, food, and fiber purposes. The seed provides oil rich in omega-3 fatty acids, digestible proteins, and lignans (Singh et al., 2011).

Functional Fiber. Nondigestible carbohydrates that have been isolated, extracted, or manufactured, and have been shown to have beneficial physiological effects in humans (Gropper & Smith, 2012).

Gastrointestinal Tract. Extends from the mouth to the anus and includes the oropharyngeal structures, esophagus, stomach, small intestine, large intestine, rectum, and anus. Primary roles are to (1) extract micronutrients, protein, carbohydrates, lipids, water, and ethanol from ingested foods and beverages; (2) absorb necessary micronutrients; and (3) serve as a physical and immunologic barrier to microorganisms, foreign material, and potential antigens consumed with food or formed during the

passage of food. Participates in regulatory, metabolic, and immunologic functions that affect the entire body (Mahan et al., 2012).

Groats. Hulled kernels of cereal grains (Skrabanja et al., 2001).

Lignan. A woody fiber found in the stems and seeds of fruits and vegetables and in the bran layer of cereals; because of conjugated double bonds, is an excellent antioxidant; some, such as that found in flaxseed, have phytoestrogen activity (Mahan et al., 2012).

Microbiota. The totality of microorganisms associated with a given environment (Scott et al., 2008).

Obesity. A body mass index of ≥ 30 kg/m² and characterized by low-grade inflammation (Centers for Disease Control and Prevention, 2014.)

Pathogenic Bacteria. Organisms capable of causing disease in humans, animals, plants, or other microorganisms (Mahowald et al., 2009).

Prebiotic. A nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson et al., 2004).

Probiotic. A live microorganism which beneficially affects the host by improving intestinal microbial content to inhibit gram negative enteric bacterial growth (Quigley 2010).

Pseudocereal. Plants that do not belong to the grass family but produce fruits and seeds used as flour for bread and other staples (Mikulikova & Kraic, 2006).

Resistant Starch. Starch that resists digestive enzyme action and reaches the colon; a starch encased in a nondigestible plant seed coat or modified by cooking or processing can be resistant (Mahan et al., 2012).

CHAPTER 2. LITERATURE REVIEW

Obesity

Obesity occurs when energy intake is greater than energy expenditure and is classified as having a Body Mass Index (BMI) of ≥ 30 kg/m² and an excess percentage of body fat; over 24% in males and over 35% in females (Centers for Disease Control and Prevention, 2014). Results from the National Health and Nutrition Examination Survey (NHANES) conducted in 2009-2010 revealed that 35.7% of adults and almost 17% of youth in the United States are obese (Ogden et al., 2012).

The prevalence of obesity continues to rise in the United States and has become a major public health concern. Between 1980 and 2000, obesity rates doubled among adults. This increase appears to occur among all age groups, genders, and racial/ethnic groups. In addition, according to The Behavioral Risk Factor Surveillance System (BRFSS) of 2011, all states reported an obesity prevalence of 20% or greater. Locally, the state of North Dakota had a 29.1% prevalence rate of obesity (Centers for Disease Control and Prevention, 2014).

Obesity is the leading risk factor for medical conditions such as hypertension, high cholesterol, type two diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis, respiratory disorders, and certain cancers including epithelial, breast, and colon cancers. While most of the diseases related to obesity are considered preventable, most people do not practice health behaviors that prevent obesity (Centers for Disease Control and Prevention, 2014).

Microbial Content in the Gastrointestinal Tract

Medical researchers continue to investigate the factors that contribute to obesity including diet, physical activity, genetic composition, and the microbial content of the gastrointestinal (GI) tract (Harris et al., 2011). Optimal function of the human GI tract is crucial

for digestion and efficient utilization of nutrients. The large intestine is responsible for activities such as the fermentation of carbohydrates as well as short chain fatty acid production by bacteria (Macfarlane & Macfarlane, 2011). The large intestine has the highest density of microorganisms compared to all other organs of the body, with the most common microorganisms belonging to the phyla *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Enterobacteriaceae*. Depending on the host's dietary intake, these bacterial populations can vary in abundance and how influential they can be (Eckburg et al., 2005).

The microbial content in the GI tract of obese individuals contained species belonging to *Bacteroidetes* decreased, while those belonging to *Firmicutes* are increased (Ley et al., 2005; Santacruz et al., 2009, 2010). After diet therapy and subsequent weight loss, researchers have found that *Bacteroidetes* populations increased while *Firmicute* populations decreased. Furthermore, scientists observed an increase in the variety of beneficial *Actinobacteria* and a decrease in detrimental *Enterobacteriaceae* (Ley et al., 2005; Santacruz et al., 2009, 2010).

Bacteroidetes include species from the genus *Bacteroides*, which are the most commonly found bacteria in the colon (Wexler, 2007). *Bacteroides* ferment carbohydrates to provide energy for the host. *Bacteroides* that reside in the colon have also been shown to interact with the immune system by supporting the development of gut-associated lymphoid tissues.

Firmicutes that are beneficial to health in the human GI tract are the *Lactobacillus* species, which help to relieve conditions such as diarrhea and irritable bowel syndrome (Quigley, 2010). They are also known for contributing to the maintenance of gut mucosa immunity by generating SCFA. The energy generated from SCFA is used in the colonic mucosa for the growth of healthy intestinal epithelial cells. SCFA may also cause tonic contractions, which

inhibit peristaltic intestinal activity and result in an overall increase in the fluid flow through the large intestine causing a reduction in fecal transit time (Cherbut, 2003).

The most common genus of *Actinobacteria* is *Bifidobacterium*. *Bifidobacterium* ferment complex carbohydrates including oligosaccharides in the GI tract (Ventura et al., 2007). Their proliferation acts as protection against other detrimental bacterial pathogens. Species including *Bifidobacterium breve* and *Bifidobacterium longum* have been identified for their roles in stimulating beneficial bacterial growth and carbohydrate fermentation.

Enterobacteriaceae include normal flora species that are protective against incoming GI pathogens (Janda & Abbott 2006). *Salmonella*, *Escherichia coli*, and *Shigella* are commonly known for causing GI upset. *Enterobacteriaceae* is commonly found in the human colon in low prevalence, however, as these species become overgrown in the colon or infect the host, they can cause severe cases of vomiting and diarrhea.

***Enterobacteriaceae* in the GI Tract**

Enterobacteriaceae are often considered detrimental species of bacteria when found in excess in the human colon. These particular species, however, are able to proliferate in the colon in low prevalence without causing immediate harm or distress such as vomiting or diarrhea (Janda & Abbott 2006). *Enterobacteriaceae* may also be related to other conditions, such as obesity, due to a mechanism which may increase intestinal permeability (Santacruz et al., 2010; Karlsson et al., 2012).

The balance of *Enterobacteriaceae* in the GI tract is affected by high fat intake, as this may cause an inflammatory response (Cani et al., 2008). Lipopolysaccharides exist on the outer membrane of *Enterobacteriaceae* and may also act as an inflammatory agent. High fat diet intake may compromise the integrity of the intestinal epithelial cell tight junction regulation,

which can then lead to the lipopolysaccharides leaking out from the GI tract into the blood stream (Amar et al., 2011). Lipopolysaccharides in the blood can cause further inflammation as well as metabolic endotoxemia. *Enterobacteriaceae* may be translocated from the GI tract into other body tissues, such as adipose tissue, which may then be released into the blood stream and cause further chronic tissue inflammation.

Higher prevalence of *Enterobacteriaceae* in the GI tract may be more prevalent in those who are obese compared to those who are normal weight. Santacruz et al., (2010) observed potential relationships between gut microbiota and body weight in pregnant women. Fifty pregnant women were recruited for the study. Thirty-four of the women were classified as normal weight per pre pregnancy BMI, while sixteen of the women were classified as overweight. Fecal samples were collected at 24 weeks of pregnancy. Using real-time quantitative polymerase chain reaction (RT-qPCR) to analyze the bacteria present, the results showed that *Enterobacteriaceae* were found at an increased rate in the women classified as obese when compared to the women classified as normal weight, specifically *Escherichia coli* ($p = 0.045$) in those with excess weight gain. They concluded that gut microbiota composition was related to body weight during pregnancy, which might be of relevance to the management of the health of women and infants. The researchers suggested that the possibility for management of body weight and of the nutritional status of pregnant women through modification of the intestinal microbiota may warrant further investigation (Santacruz et al., 2010).

Karlsson et al., (2012) collected fecal samples from twenty male and female children classified as overweight or obese by their BMI (17.6-25.8 kg/m²). Twenty male and female children with a normal BMI (13.6-17.2 kg/m²) had fecal samples collected and served as a control group. The ages of the children ranged between about 4-5 years. Using RT-qPCR,

Enterobacteriaceae were found to be significantly higher in obese and overweight children ($p = 0.036$) when compared to children with a BMI within the normal range (Karlsson et al., 2012).

Xiao et al., (2013) found that a decrease in *Enterobacteriaceae* in the GI tract and changes in the gut microbiota through dietary intervention may enhance intestinal integrity. A total of 89 male (57) and female (32) Chinese participants aged 25-55 years with a BMI of 28 or greater completed a self-controlled clinical trial consisting of a 9-week diet intervention followed by a 14 week maintenance period. The participants were asked to consume a diet based on whole grains, traditional Chinese medicinal foods, and prebiotics. The average weight loss was 5.79 ± 4.64 kg with significant reductions in *Enterobacteriaceae* prevalence, as well as improvements in lipid profiles, blood pressure, and insulin sensitivity. Gut permeability was also decreased compared to the participants baseline measurements using a lactulose/mannitol ratio (Xiao et al., 2013).

Weight Loss in Obesity Related to Bacterial Content in the GI Tract

During weight loss, the ratio of beneficial bacteria compared to pathogenic bacteria appears to shift. Researchers have observed an increase in beneficial bacteria and a decrease in pathogenic bacteria directly related to weight loss (Ley et al, 2005; Santacruz et al, 2009).

A study performed by Ley et al. (2006) found that *Bacteroidetes* populations are lower in obese individuals, but increase as weight loss continues on low-calorie diets. For one year, the researchers followed 12 obese subjects participating in a weight-loss program, which included both men and women from 21-65 years of age. The participants were randomly assigned to either a fat restriction group (30% calories from fat) or a carbohydrate restriction group (25% calories from carbohydrates). Over the course of the year, the researchers monitored gut microbes by sequencing 16S ribosomal RNA genes from morning stool samples at 0 week, 12 weeks, 26

weeks, and 52 weeks. Prior to the diet interventions, the subjects had fewer *Bacteroidetes* and more *Firmicutes* than a lean control group. As weight loss progressed, the researchers observed an increase in *Bacteroidetes*, while the number of *Firmicutes* decreased in both diet-restriction types. The observed changes in bacterial populations also correlated with loss of body weight. The researchers suggest that manipulation of the gut microbial communities could be an approach to obesity treatment (Ley et al., 2006).

Santacruz (2009) found that an intervention with a reduction in energy intake and an increased energy expenditure impacts microbial composition in the colon. Participants included 36 adolescents (18 female, 18 male, mean age 14.5 years) classified as overweight or obese. For ten weeks, subjects were asked to consume a 10-40% calorie restricted diet with an increase in physical activity (15-23 kcal/kg body weight/week), established according to degree of obesity and regular physical activity prior to the study to promote weight loss. The subjects kept food diaries and also provided a fecal sample prior to their participation in the study and also at the completion of the study for bacterial analysis. After the intervention was completed, the researchers divided the subjects into two groups for statistical analysis based on the amount of weight each subject lost during the course of the study. The low weight loss group consisted of 13 subjects that demonstrated a weight loss of less than 2 kg after ten weeks of study intervention. The high weight loss group consisted of 23 subjects that lost over 4 kg of weight after 10 weeks of study intervention. Results of the microbial analysis performed on the fecal samples using a quantitative real-time polymerase chain reaction method (RT-qPCR) found that all participants had significantly increased beneficial bacterial populations such as *Bacteroides fragilis* and *Lactobacillus* spp. in the colon. Researchers concluded that the reduction of energy intake and the increase of energy expenditure positively correlated with the body weight loss in

adolescents. They also observed a positive correlation between weight loss and the composition of beneficial microbial content (Santacruz et al., 2009).

Fiber and the Fiber Content of Flaxseed and Buckwheat

Fiber is recognized for its role in promoting satiety by decreasing gastric emptying rate and increasing fecal bulk (Brownawell et al., 2012). Fiber is classified as dietary fiber or functional fiber. Both categories of fiber consist of carbohydrates that are not digestible by enzymes of the GI tract.

Functional fiber in particular has been shown to have beneficial physiological effects in humans (Bengmark & Martindale 2005). Many functional fiber sources are also fermentable including inulin, pectin, gums, β -glucan, and resistant starch (RS). As fermentation in the colon occurs, the growth of beneficial bacteria species increase and pathogenic populations decrease.

Skrabanja et al. (2001) performed research to identify the nutritional properties of buckwheat products, including post-prandial satiety, resistant starch analysis, and starch hydrolysis involving ten healthy human subjects aged 23-53 years. Following an overnight fast, the subjects were given a breakfast on three separate occasions that would include one of the diet treatments in a random selective order. The treatments included boiled buckwheat groats, bread baked with 50% buckwheat groats, and bread made from white wheat flour. The highest proportion of resistant starch was found in the boiled buckwheat groats followed by the 50% buckwheat groat bread product. Subject's satiety scores for buckwheat products compared to processed white flour products were reported to be significantly higher. Researchers concluded that the high resistant starch contents of dietary fiber found in buckwheat resulted in high post-prandial satiety (Skrabanja et al., 2001).

Fiber in flaxseed was examined using flaxseed in a bread product and in a drink mixture. The drink mixture included water, blackberry syrup, and flaxseed fiber powder, which was consumed 30 minutes prior to breakfast. Subjects included 17 young human subjects, both men and women, to follow three separate diet protocols for seven days with a greater than one week washout between each diet. The treatments included a diet low in fiber, a diet including a flaxseed fiber drink three times per day, and a diet with flaxseed fiber bread three times per day. Each treatment was accompanied by a standardized daily diet provided from the treatment center. The flaxseed fiber diet treatments provided 7.5 g/10 megajoules of dietary fiber per treatment, while the low fiber diet provided 12 g/10 megajoules of modified corn starch per treatment. Fecal excretion of fat and blood lipid concentration was compared in each treatment group. Daily consumption of dietary fiber from flaxseed significantly increased fecal excretion of fat and reduced cholesterol concentrations in the blood when compared to a low fiber diet (Kristensen et al., 2012). The observed affect of flaxseed may be related it's fiber and prebiotic activity.

Prebiotics

A prebiotic is an undigestible food ingredient that beneficially affects the host by selectively stimulating growth and/or activity of bacteria in the colon, and improves host health (Gibson et al., 2004). The substrate must first demonstrate that it cannot be hydrolyzed in the upper GI tract and must resist gastric acidity. Secondly, it must show fermentation by intestinal microbiota and selectively stimulate growth or activity of bacteria that are considered beneficial to the host's health and well-being. Lastly, it must alter intestinal microflora towards a healthier composition.

Wang et al. (2012) tested the potential health promoting effects of fermented milk that contained a combination of prebiotic and probiotic substrates. Researchers used both human and animal models to observe the changes in intestinal microbiota. One hundred healthy adult volunteers (50 male, 50 female, mean age 35 years) were divided randomly into two treatment groups in order to analyze fecal bacteria from both the control and test groups. The test group consumed 480 g of fermented milk, which was supplemented with the probiotics *Lactobacillus acidophilus* and *Bifidobacterium lactis* as well as the prebiotic isomaltooligosaccharides for 14 days. Wang also used 40 male BALB/c mice divided into four treatment groups including a control group that received sterile water and three milk fermentation groups; low-dose (0.4 g/10 g of body weight), medium-dose (0.8 g/10 g of body weight), and high-dose (2.4 g/10 g of body weight). The mice received the treatment for 14 days through a gastric tube. Fecal sample analysis from the human subjects that consumed the fermented milk showed a significant increase in *Lactobacillus* and *Bifidobacteria* when compared to the control group. The mice also showed a significant increase in *Bifidobacteria* and *Lactobacillus* in the medium-dose and high-dose groups compared to the control group. Wang concluded that the fermented milk treatments containing both probiotics and prebiotics may have contributed to the improved intestinal health of both human and BALB/c mice. (Wang et al., 2012).

In an effort to replace antibiotics in animal feed, prebiotic and probiotic additives have been proposed as a treatment for healthy newborn calves. Roodposhti and Dabiri studied the effects of feeding whole milk containing prebiotics, probiotics, or a combination to 32 female Holstein calves at two weeks of age. The researchers assessed average daily gain of weight in the calves relative to *Escherichia coli* counts and immune status without the use of conventional antibiotics. Each of the calves received whole milk treatments with a specific additive. Calves

were assigned randomly to one of four treatment groups including a group without additive, a probiotic additive, a prebiotic additive, and both probiotic and prebiotic additives. They received each treatment twice daily for 60 days. Their goal was to compare each treatment group's response in average daily weight gain, fecal *Escherichia coli*, white blood cell count, plasma immunoglobulin G1 level, and cell-mediated immune response. Although Roodposhti and Dabiri did not observe any significant changes in white blood cell count, plasma immunoglobulin G1 concentrations, or cell-mediated responses between groups, the researchers found that the use of probiotic, prebiotic, and probiotic-prebiotic combination reduced *Escherichia coli* in the feces. The average daily gain of weight in the calves that had consumed the prebiotic, probiotic, and prebiotic-probiotic combination were significantly higher than the control during the final three weeks of the study. The researchers concluded that a decrease in fecal *Escherichia coli* using dietary prebiotics and probiotics had a positive effect on a healthy weight gain and growth (Roodposhti & Dabiri, 2012).

Buckwheat and Prebiotic Activity

Buckwheat is a pseudocereal grain that does not contain gluten (Mikulikova & Kraic, 2006). Additionally, buckwheat has been identified as having exceptional prebiotic capability. In a study comparing a variety of pseudocereals including quinoa, millet, and sorghum, it was clear that buckwheat contained the highest content of resistant starch (37.9 ± 3.6 g/kg dry weight basis) suggesting it has strong prebiotic functionality.

Prebiotic activity for buckwheat was measured in a research study using twenty, 12-week-old Wistar Hannover Rats. The rats were split into two groups of ten. The first group was used as the control and was fed generic mouse chow and the second group was fed cooked buckwheat. Each group was fed for 30 days. Préstamo identified more *Lactobacillus* and

Bifidobacterium in the intestines of the rats that were fed the buckwheat diet. Furthermore, they were unable to identify beneficial bacteria species including *Lactobacillus plantarum* and *Bifidobacterium lactis* in the control group. The buckwheat group had fewer pathogenic bacteria including *Enterobacteria*. Researchers concluded that buckwheat stimulated the growth of beneficial bacteria and altered intestinal microflora towards a healthier composition (Préstamo et al., 2003).

Flaxseed and Prebiotic Potential

Flaxseed is an oilseed crop and is recognized for being high in dietary fiber (20-25%) and 45-52% omega-3 fatty acids (Singh et al., 2011). It is also an excellent source of secoisolariciresinol diglucoside (SDG), which is a phytoestrogen lignan with high antioxidant activity (Hao & Beta, 2012). Park and Velasquez found the effects of SDG in flaxseed powder supplementation to provide beneficial effects including reduced body weight, reduced fat accumulation, and blood lipid profile improvement. Eight-week old Sprague-Dawley male rats were divided into four groups of eight animals each. The four treatment groups included a control diet (NC), a control diet with 0.02% SGD lignan-enriched flaxseed powder (NCL), a high-fructose and fat diet (HFD), and a high-fructose and fat diet with 0.02% SGD lignan-enriched flaxseed powder (HFDL). The average body weight of the high-fructose and fat diet group was significantly higher than the other three groups. The lipid profile of the rats in the HFDL group had lower concentrations of cholesterol and triglycerides than the HFD group ($p < 0.05$).

Upon extensive review of literature, there was no research related to flaxseed and prebiotics that could be found. The high dietary fiber content of flaxseed indicates flaxseed may have prebiotic potential (Kristensen et al., 2012).

Summary

Traditional factors recognized as causes for obesity include high calorie diet, decreased physical activity, and genetic predisposition (Harris et al., 2011). However, an emerging factor associated with obesity includes the microbial content of the GI tract. Bacterial interactions may cause or inhibit obesity depending on the ratio of beneficial to pathogenic bacteria in the colon (Ley et al, 2005; Santacruz et al, 2009). *Enterobacteriaceae* in the GI tract may be more prevalent in those who are obese compared to those who are normal weight (Santacruz et al., 2010). Functional fibers are fermentable in the colon, which stimulates the growth of bacteria (Bengmark & Martindale, 2005). Flaxseed and buckwheat are plants with a high fiber content, which indicate they may proliferate bacteria in the GI tract (Skrabanja et al., 2001; Kristensen et al., 2012).

CHAPTER 3. METHODS

Animals

Approval from the Institutional Animal Care and Use Committee (IACUC) of North Dakota State University (NDSU) was obtained prior to the initiation of this study (#A13019). Seventy-two C57BL/6J male mice from the Jackson Laboratory in Bar Harbor, Maine were randomly assigned to one of six treatment groups for a total of 12 mice per group. This animal model is prone to developing obesity after being fed a high-fat diet (Wang and Liao, 2012; Rabot et al., 2010). Male mice were the chosen subjects due to the SGD content of flaxseed, which is a phytoestrogen lignan that may interfere with the estrogen hormone in female mice (Mahan et al., 2012, Hao & Beta, 2012). The mice were approximately six weeks old when received, and were then acclimated to the laboratory conditions for one week prior to dietary treatment. Each experimental diet was fed for a total of eight weeks.

Animals were kept individually in plastic-bottom cages at a controlled room temperature (22-25°C) and humidity (42-55%) with a 12-hour light/dark cycle in the Animal Nutrition and Physiology Center at NDSU. Fresh food and water was provided daily. Bedding was changed weekly. Animals had access to food and water *ad libitum*, with exception to the pair-fed lean treatment group, as described in diet treatment groups. Sterile bedding upon arrival and throughout the study tested negative for *Enterobacteriaceae*. After eight weeks of experimental diet feeding, the mice were euthanized using carbon dioxide gas, then exsanguinated by anterior cardiac puncture according to IACUC's approved procedures.

Treatment Groups

All mice were randomly assigned to one of six treatment groups; Group 1 (45% Kcal fat, control), Group 2 (45% Kcal fat, 10% whole flaxseed), Group 3 (45% Kcal fat, 6% defatted

flaxseed), Group 4 (45% Kcal fat, 4% flaxseed oil), Group 5 (45% Kcal fat, 10% buckwheat), and Group 6 (control diet, 16% Kcal fat, pair-fed to group 2). The amount of defatted flaxseed and flaxseed oil was adjusted based on lignan and n-3 fatty acid content in the whole flaxseed supplementation diet. Food consumption of the mice in Group 2 was measured daily by grams and the same amount was provided to Group 6 in grams.

Flaxseed and Buckwheat Diet Formulation

The flaxseed was obtained from local suppliers in the Fargo, North Dakota area and combined to prepare flaxseed supplemented diets. The whole flaxseed was milled to a particle size of 30 mesh. The whole milled flaxseed was subjected to hexane extraction to produce both defatted flaxseed and the flaxseed oil. After the hexane was removed, the remaining defatted flaxseed was dried at room temperature until all solvent was removed. The remaining hexane extracted flaxseed oil was used to prepare served as the flaxseed oil supplemented diet. North Dakota grown whole buckwheat was donated by Dr. Darrin Haagenson, in the Department of Agricultural and Biosystems Engineering at NDSU.

TestDiet® laboratories formulated the experimental diet in the form of pellets for animal consumption. Each of the six diet treatments were dyed with a unique color to ensure correct group identification for feeding. Vitamin E was not added to any of the diet treatments in order to remove any additional antioxidant effects. The nutritional profile of the low fat diet contained 18.8% kcal from protein, 16.4% kcal from fat, and 64.9% kcal from carbohydrates with 5% from fiber. The nutritional profile of the high fat diets contained 18% kcal from protein, 45% kcal from fat, and 37% kcal from carbohydrates with 6% from fiber. Pellets from each of the diet treatment groups were randomly tested for presence of *Enterobacteriaceae* prior to treatment. All of the food pellets tested negative for presence of *Enterobacteriaceae*.

Body Weight and Diet Consumption

Body weight in grams of each mouse was measured pre treatment and also on a weekly basis throughout the eight weeks of study. Food consumption in grams was measured and monitored on a weekly basis to ensure the consumption of each diet. Daily consumption in grams was measured from the 10% whole flaxseed treatment group to adequately pair-feed the lean diet treatment group.

Fecal Sample Collection

Fecal samples were collected from each mouse at week 0 (pre treatment) with a one week acclimation period, and again eight weeks later at week 9 (post treatment). Bedding removal and sterilization of cages with 70% ethanol occurred prior to fecal sample collection. Cages were left bedding free for 12 hours to allow for fecal sample collection. Collected fecal samples were weighed and then processed with 10 mL sterile phosphate based saline (PBS) for content homogenization. Collected fecal samples from each mouse were combined with fecal samples from another mouse from their treatment group to manage agar plate storage and supply. The same combination was used both pre and post treatment.

***Enterobacteriaceae* Isolation and Identification**

A one mL aliquot was removed from each feces homogenate and inoculated onto a sterile MacConkey agar plate, then incubated for 48 hours at 37°C to culture *Enterobacteriaceae*. Plates were then streaked for isolation onto a fresh MacConkey agar plate with a sterilized inoculum loop. Isolated streaks were incubated for 48 hours at 37°C.

Another one mL aliquot was also removed from each feces homogenate to undergo a serial dilution and was placed into a tube containing 9 mL of PBS. This dilution process was repeated up to five times. Post treatment feces serial dilutions also included 10⁴ and 10⁵ due to

increased bacterial proliferation following diet supplementation. The colony forming units (CFU) were counted on each MacConkey agar plate. Plates were then streaked for isolation onto a fresh MacConkey agar plate with a sterilized inoculum loop. Isolated streaks were incubated for 48 hours at 37°C.

Enterobacteriaceae was identified using the Remel RapID™ ONE System. The Remel RapID™ ONE System uses a tray, which contains multiple cavities. Each of the cavities contains individual reactive ingredients. When the cavities are inoculated with an *Enterobacteriaceae* sample of interest, the following reaction allows for the identification of *Enterobacteriaceae*. The resulting color change was used to determine positive or negative identification due to change in pH and enzymatic hydrolysis.

After eight weeks of diet supplementation, the cecum was removed from each mouse and homogenized using a Stomacher® 400 homogenizer with 10 mL PBS. Processing occurred in the same manner as fecal sample bacterial isolation for *Enterobacteriaceae* described above.

Statistical Analyses

An average was used to compare pre and post weight among treatment groups as well as weight gain and food intake among treatment groups. Chi Square for homogeneity was used to compare the differences among diet treatment groups including *Enterobacteriaceae* frequency of detection as well as high and low prevalence of *Enterobacteriaceae* between weeks 0 and 9 of treatment. The total of CFU calculation was completed by adding the estimated observed number of colonies with the dilution factor for each treatment group. A Dunn's multiple comparison was used to compare the means of CFUs of *Enterobacteriaceae* among post treatment fecal samples. An ANCOVA was used to compare weight at post treatment to the variables including the diet treatment groups fed a 45% high fat diet, weight at pre treatment, average weekly intake,

Enterobacteriaceae present in pre fecal samples, *Enterobacteriaceae* present in post fecal samples, and *Enterobacteriaceae* present in the cecum. Any significant results from the analysis were reported with an alpha level of 0.05. All data were analyzed using the statistical analysis program SAS 9.3.

CHAPTER 4. RESULTS AND DISCUSSION

Weight and Food Intake

The average pre treatment weight among diet groups was significantly different ($p < 0.013$), as well as the average post treatment weight ($p < 0.0001$). The weight gain between pre and post treatments was also significantly different ($p < 0.0017$) (Table 1). The mice from the whole flaxseed (WF), high fat (HF), and buckwheat (BW) groups had the most weight gain after the eight-week treatment period. The weight of low fat (LF) treatment group at post treatment was significantly lower than all of the treatment groups except the flaxseed oil (FO) group (Table 1). The difference in weight gain between the LF treatment groups compared to the defatted flaxseed (DF), buckwheat (BW), HF, and WF groups is most likely related to the difference in percentage of calories from fat between the diets.

The average weekly food intake was significantly different among treatment groups ($p < 0.007$) (Table 1), which may indicate that food intake could have had an effect on the differences in weight gain between dietary treatments. However, in an ANCOVA created to compare the variables that may impact weight changes, we were unable to conclude weekly intake may have an effect on weight change.

We compared the weight of our C57BL/6J mice at fifteen weeks old to the average C57BL/6J mouse at fifteen weeks old from the Jackson laboratories. The average fifteen week C57BL/6J mouse fed a standard diet with 10% fat from the Jackson laboratories in their own independent study weighed at least 2 g less than the WF mice in this study fed a 45% fat diet at fifteen weeks of age (Tables 1 and 2). The difference in calories from fat between the diet treatment groups is most likely related to this observation of difference in average weight gain. The mice in LF diet group, fed 16% fat, had a lower average weight gain than the C57BL/6J

mice from the Jackson laboratories fed a standard 10% fat diet. The mice in our LF diet treatment, however, were not fed ad libitum and were instead pair fed to the WF diet treatment, which is likely the cause of the difference in average weight gain. The greater percentage of weight gain of mice fed a 45% fat diet compared to the LF group indicates that these mice in our current study were obese (Table 1).

Table 1. Weight at pre and post treatments, weight gain, percent weight gain, and weekly food intake throughout 8 weeks of treatment*

n	Group	Weight at Pre Treatment (g)	Weight at Post Treatment (g)	Weight Gain (g)	Weight Gain (%)	Weekly Food Intake (g)
12	High Fat	19.88 ± 1.32	33.80 ± 3.41	13.92 ± 2.37	142.50	25.23 ± 5.81
12	Whole Flaxseed	21.10 ± 1.08	39.27 ± 5.34	18.17 ± 3.21	165.56	27.53 ± 4.01
12	Defatted Flaxseed	19.81 ± 1.64	31.55 ± 3.78	11.74 ± 2.71	133.01	21.68 ± 1.54
12	Flaxseed Oil	20.12 ± 1.07	29.62 ± 2.16	9.50 ± 1.62	124.87	20.25 ± 0.75
12	Buckwheat	19.81 ± 1.58	32.48 ± 5.02	12.67 ± 3.30	136.93	24.69 ± 3.61
11	Low Fat	19.61 ± 2.25	23.72 ± 2.51	4.11 ± 2.06	†	27.00 ± 3.91
P value		0.013	0.0001	0.0017		0.007

*Data were reported as mean ± standard deviation.

† The LF diet (16% fat) was used as the comparison for control as the other diet treatment groups were fed at 45% fat diet.

Jackson Laboratories, where our mice originated, supplies average body weight information for their C57BL/6J mice throughout their lifecycle when fed a 10% fat diet and 60% fat diet (The Jackson Laboratories). In our current study, at fifteen weeks of age, the average weight gain of the WF mice was greater than the average weight gain of a C57BL/6J mouse of the same age fed a 60% high fat diet (Tables 1 and 2). Our C57BL/6J mice fed a 45% high fat diet had a similar average weight gain compared to the C57BL/6J mice from the Jackson Laboratory (Tables 1 and 2). It is likely to assume that the threshold of weight gain for these mice may reach its peak with a 45% fat diet. Both 45% fat and 60% fat diets would induce

obesity in this animal model. The similarities between the average weight gain of our HF and BW diets compared to the Jackson Laboratory 60% fat diet treatment is also likely related to this (Tables 1 and 2).

Table 2. Average weight of C57BL/6J mice at six and fifteen weeks of age

	Average Weight at Six Weeks of Age (g)	Average Weight at Fifteen Weeks of Age (g)	Average Weight Gain (g)
Jackson Laboratories (10% fat diet)	21.10 ± 1.50	29.70 ± 2.20	8.60 ± 1.85
Jackson Laboratories (60% fat diet)	21.70 ± 1.50	36.20 ± 4.20	14.50 ± 2.85
Current Study (45% fat diet)	20.14 ± 1.34	33.34 ± 3.94	13.20 ± 2.24

Enterobacteriaceae in the Feces and Cecum

The diversity of *Enterobacteriaceae* identified in the feces homogenate and cecum included *Escherichia coli*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Enterobacter amnigenus*, *Serratia marcescens*, *Serratia liquifaciens*, *Shigella*, *Cronobacter sakazakii*, and *Pantoea agglomerans*. The post fecal samples with the most diversity of *Enterobacteriaceae* overall were observed to be in the WF with five species and BW with four species (Table 3). These results suggest that the high fermentable fiber content of flaxseed (Kristensen et al., 2012) led to the increased diversity of *Enterobacteriaceae* through fermentation (Bengmark & Martindale, 2005). Buckwheat supplementation also shed *Enterobacteriaceae* in the feces and cecum, which is in agreement with Prestamo (2003) regarding supplementation of buckwheat into the diet and shedding of *Enterobacteriaceae*.

Table 3. Diversity of *Enterobacteriaceae* in pre fecal, post fecal, and cecum samples between treatment groups

Group	Pre Fecal Samples	Post Fecal Samples	Cecum Samples
High Fat	<i>Serratia spp.</i>	<i>S. marcescens</i>	<i>S. marcescens</i> <i>P. agglomerans</i>
Whole Flaxseed	<i>Serratia spp.</i> <i>E. amnigenus</i> <i>E. cloacae</i>	<i>S. marcescens</i> <i>E. cloacae</i> <i>E. aerogenes</i> <i>P. agglomerans</i> <i>C. sakazakii</i>	<i>S. marcescens</i> <i>P. agglomerans</i>
Defatted Flaxseed	<i>Serratia spp.</i>	<i>S. marcescens</i> <i>E. cloacae</i> <i>P. agglomerans</i>	<i>S. marcescens</i> <i>E. aerogenes</i>
Flaxseed Oil	<i>Serratia spp.</i>	<i>S. marcescens</i>	<i>S. marcescens</i>
Buckwheat	<i>Serratia spp.</i> <i>S. liquefaciens</i> <i>E. amnigenus</i> <i>E. cloacae</i> <i>P. agglomerans</i>	<i>S. marcescens</i> <i>E. aerogenes</i> <i>P. agglomerans</i> <i>E. cloacae</i>	<i>S. marcescens</i> <i>E. aerogenes</i> <i>E. amnigenus</i> <i>P. agglomerans</i>
Low Fat	<i>Serratia spp.</i> <i>E. amnigenus</i> <i>P. agglomerans</i> <i>Shigella</i>	<i>E. aerogenes</i> <i>E. cloacae</i> <i>P. agglomerans</i>	<i>S. marcescens</i> <i>E. aerogenes</i> <i>E. cloacae</i> <i>E. coli*</i>

*The data of one mouse from the lean diet group with *E. Coli* has been omitted from the data analysis due to displaying characteristics of illness and distress.

There was no significant difference in *Enterobacteriaceae* prevalence in pre treatment fecal samples among treatment groups ($p < 0.4418$). This indicated that each of the groups had a similar frequency of *Enterobacteriaceae* prevalence in their feces prior to dietary treatment, which is favorable (Table 4). The treatment groups with the lowest total CFU pre treatment were the HF, WF, and FO groups. The treatment groups with the highest CFU were in the DF, BW, and LF groups (Table 5). A low and high prevalence for the pre treatment samples was not calculated, as these results did not appear to be approaching significance.

Table 4. Frequency of detection of *Enterobacteriaceae* in pre and post treatment fecal samples

Diet Group	Absence of <i>Enterobacteriaceae</i>		Presence of <i>Enterobacteriaceae</i>	
	Pre	Post	Pre	Post
High Fat	1	2	5	4
Whole Flaxseed	1	0	5	6
Defatted Flaxseed	1	0	5	6
Flaxseed Oil	3	3	3	3
Buckwheat*	0	0	5	6
Low Fat	1	2	4	3

$p < 0.4418$ probability of homogeneity across treatment groups in the pre treatment fecal samples. Fecal samples from each mouse were combined with fecal samples from another mouse from their treatment group. The same combination was used both pre and post treatments.
 $p < 0.0942$ probability of homogeneity across treatment groups in the post treatment fecal samples. Fecal samples from each mouse were combined with fecal samples from another mouse from their treatment group. The same combination was used both pre and post treatments.
*1 sample missing due to misplacement.

The presence of *Enterobacteriaceae* in post treatment fecal samples was also not significantly different among dietary treatment groups ($p < 0.0942$, Table 4). Because the results appeared to be approaching significance, the prevalence of *Enterobacteriaceae* was then categorized as either low or high. High prevalence was identified as samples diluted to 10^1 or higher and that continued to grow a species of *Enterobacteriaceae*. Low prevalence was identified as samples with *Enterobacteriaceae* present only when undiluted or with no *Enterobacteriaceae* detected. The high and low prevalence of *Enterobacteriaceae* in post treatment fecal samples were significantly different among treatment groups ($p < 0.0033$, Table 6). The total CFU in the post treatment fecal samples were all significantly different from each other between treatment groups (Table 5). HF and FO had the lowest prevalence and CFU in

post treatment fecal samples, with 100 CFU and 10 CFU respectively. These were also the treatment groups with a low fermentable fiber content. WF, DF, and BW had the highest prevalence and CFU in post treatment fecal samples with 105,000 CFU, 102,210 CFU, and 200,200 CFU respectively (Tables 5 and 6). This indicates that *Enterobacteriaceae* was most often present in fecal samples following the diet treatment with a high fermentable fiber content (Singh et al., 2011; Mikulikova & Kraic, 2006; Kristensen et al., 2012; Prestamo et al., 2003). Upon extensive review of literature, we were unable to find other research similar to the current study using mice as an obesity model comparing the prevalence of *Enterobacteriaceae* in the GI tract.

Table 5. Total colony forming units (CFU) of *Enterobacteriaceae* per gram in pre fecal, post fecal, and cecum samples

Group	Total CFU in pre fecal samples	Total CFU in post fecal samples*	Total CFU in cecum samples
High Fat	120	100	1 x 10 ⁷
Whole Flaxseed	230	105,000	4 x 10 ⁷
Defatted Flaxseed	1110	102,210	2 x 10 ⁷
Flaxseed Oil	120	10	1 x 10 ⁷
Buckwheat	2020	200,200	1 x 10 ¹⁰
Low Fat	3110	2020	3 x 10 ⁷

Total CFU completed by adding the estimated observed number of colonies with the dilution factor for each treatment group.

*A Dunn's multiple comparison test found that the all of the treatment groups were significantly different from each other in the post fecal samples (p < 0.05).

Table 6. Low or high prevalence of *Enterobacteriaceae* in the post treatment fecal samples

Diet Group	Low Prevalence of <i>Enterobacteriaceae</i>	High Prevalence of <i>Enterobacteriaceae</i>
High Fat	5	1
Whole Flaxseed	0	6
Defatted Flaxseed	0	6
Flaxseed Oil	5	1
Buckwheat	2	4
Low Fat	2	3

$p < 0.0033$ probability of homogeneity across post treatment fecal treatment groups. Fecal samples from each mouse were combined with fecal samples from another mouse from their treatment group. The same combination was used both pre and post treatments. High prevalence was identified as samples diluted to 10^1 or higher and that continued to grow an *Enterobacteriaceae* spp. Low prevalence was identified as samples with *Enterobacteriaceae* present only when undiluted or with no *Enterobacteriaceae* detected.

Enterobacteriaceae colonization prevalence was also measured in cecum samples as *Enterobacteriaceae* species proliferate in the GI tract (Backhed et al., 2004). *Enterobacteriaceae* present in the cecum among treatment groups was approaching significance ($p < 0.0565$, Table 7). However, when using the same method as previously described to determine high and low prevalence of *Enterobacteriaceae*, the results were significant ($p < 0.0348$, Table 8). Again, the groups with the highest prevalence of *Enterobacteriaceae* were WF and BW, which are the treatment groups with a high fermentable fiber content. The groups with the lowest prevalence of *Enterobacteriaceae* were FO and HF, which were also the groups with the lowest fermentable fiber content. Our results indicate that a potential relationship exists between high fermentable fiber diets and *Enterobacteriaceae* proliferation.

The observation can be made that *Enterobacteriaceae* found in the cecum had a similar outcome regarding the prevalence of *Enterobacteriaceae* in the fecal samples between treatment

groups. This consistency between prevalence of *Enterobacteriaceae* in both the fecal and cecum samples indicates that *Enterobacteriaceae* proliferation in the cecum had an effect on the prevalence of *Enterobacteriaceae* detected in the fecal samples.

Table 7. Frequency of detection of *Enterobacteriaceae* in the cecum

Diet Group	Absence of <i>Enterobacteriaceae</i>	Presence of <i>Enterobacteriaceae</i>
High Fat	3	3
Whole Flaxseed	1	5
Defatted Flaxseed	3	3
Flaxseed Oil	5	1
Buckwheat	0	6
Low Fat	2	3

$p < 0.0565$ probability of homogeneity across cecum sample treatment groups. Cecum samples from each mouse were combined with cecum samples from another mouse from their treatment group.

Table 8. Low or high prevalence of *Enterobacteriaceae* in the cecum

Diet Group	Low Prevalence of <i>Enterobacteriaceae</i>	High Prevalence of <i>Enterobacteriaceae</i>
High Fat	5	1
Whole Flaxseed	1	5
Defatted Flaxseed	4	2
Flaxseed Oil	5	1
Buckwheat	1	5
Low Fat	2	3

$p < 0.0348$ probability of homogeneity across treatment groups. Cecum samples from each mouse were combined with cecum samples from another mouse from their treatment group. High prevalence was identified as samples diluted to 10^1 or higher and that continued to grow an *Enterobacteriaceae* spp. Low prevalence was identified as samples with *Enterobacteriaceae* present only when undiluted or with no *Enterobacteriaceae*.

Prevalence of *Enterobacteriaceae* and Effect on Post Treatment Weight

The data were analyzed using an ANCOVA test to determine if a covariate existed that may affect our dependent variable of post treatment weight. Our original model included the covariates weight at pre treatment, diet treatment, and average weekly intake. Our results did not find any of significant differences. A second model included the covariates *Enterobacteriaceae* presence in the feces at pre treatment, feces at post treatment, and the cecum samples. These results also did not find any significant differences. A third model was created to exclude the LF diet treatment group and include only the diet treatment groups fed at 45% high fat diet. This indicated a significant difference between the diet treatment groups ($p < 0.0019$), which indicated diet treatment had an effect on post treatment weight. The model indicated a relationship existed between the prevalence of *Enterobacteriaceae* in the post treatment fecal samples and post treatment weight among the treatment groups ($p < 0.0043$). Further analysis indicated that the only dietary treatment group to have a significant effect on post treatment weight was the WF group ($p < 0.05$). The WF group was among the treatment groups with the highest prevalence of *Enterobacteriaceae* in post fecal samples (Tables 5 and 6), which may indicate a high prevalence of *Enterobacteriaceae* has an effect on weight gain (Table 1). Our results indicate there may be a potential relationship between an increase in *Enterobacteriaceae* prevalence and an increase in weight gain. The weight for the WF group at post treatment indicates that these mice were obese. This is similar to Santacruz (2010), Karlsson (2012), and Xiao (2013), who identified higher prevalence of *Enterobacteriaceae* in human obese subjects.

The ANCOVA model did not indicate any significant differences between the average weekly food intake variable and post treatment weight. This indicates that the average weekly food intake between groups did not affect post treatment weight. The ANCOVA model also did

not indicate any significant differences between the presence of *Enterobacteriaceae* in the cecum variable and post treatment weight.

General Discussion

The purpose of this research aimed to determine whether or not diets supplemented with flaxseed and buckwheat affect the prevalence of *Enterobacteriaceae* in the feces and colon as well as determining a relationship between *Enterobacteriaceae* and weight.

Support for our initial hypothesis, that flaxseed and buckwheat supplementation would increase shedding of *Enterobacteriaceae* into the feces and increase proliferation of *Enterobacteriaceae* in the cecum, was demonstrated by the significant differences in high versus low *Enterobacteriaceae* prevalence in the cecum ($p < 0.0348$) and feces ($p < 0.0033$), based on whether or not *Enterobacteriaceae* continued to grow when diluted 10^1 or higher. These findings suggest that high and low prevalence of *Enterobacteriaceae* were significantly different among diet treatment groups. WF showed the highest frequency of *Enterobacteriaceae* prevalence in both post treatment fecal and cecum samples, while the FO and HF diet treatment groups were among the lowest prevalence of frequency. To our knowledge, this is the first research that identified flaxseed having increased *Enterobacteriaceae* shedding in the feces and proliferation in the cecum. These results suggest that the high fermentable fiber content of flaxseed (Kristensen et al., 2012), lead to the increase of *Enterobacteriaceae* through fermentation (Bengmark & Martindale, 2005). The results indicated that buckwheat supplementation also proliferated *Enterobacteriaceae* in the feces and colon, which is in agreement with Prestamo (2003).

Support for our second hypothesis, we predicted the most weight gain among the high fat groups, while the groups fed with fiber from flaxseed and buckwheat would demonstrate lower

overall weight gain, is limited. The average weight gain among treatment groups throughout eight weeks of treatment was significantly different ($p < 0.0001$, Table 1). However, the groups with the highest percentage of weight gain post treatment were WF and HF. The group with the lowest percentage of weight gain post treatment was the LF treatment. The difference in weight gain between the LF treatment group compared to the DF, BW, HF, and WF groups is most likely related to the difference in percentage of calories from fat between the diets. The greater the percentage of weight gain of our treatment groups fed a 45% fat diet compared to our LF group indicates that the mice in our current study were obese (Table 1).

Support for our final hypothesis was also limited. We hypothesized that the groups with the most bacterial proliferation would be the groups with the lowest overall weight gain. Throughout eight weeks of dietary treatment, all mice fed a 45% high fat diet (HF, WF, DF, FO, and BW) gained significantly more weight than the control, LF fed mice (Table 1). However, the only diet treatment group that could be significantly related to post treatment weight was the WF group. This group had the highest prevalence of *Enterobacteriaceae* in both post fecal and cecum samples. Our results suggest that WF in relation to its shedding of *Enterobacteriaceae* in the feces had an effect on weight gain over eight weeks of supplementation. Santacruz (2010) and Karlsson (2012) found *Enterobacteriaceae* most prevalent in overweight and obese human subjects prior to weight loss, while our study indicates increase in *Enterobacteriaceae* with weight gain. Xiao (2013) also observed changes in GI microbiota following dietary supplementation and intervention including whole grains, traditional Chinese foods, and prebiotics. However, Xaio (2013) found *Enterobacteriaceae* to decrease with weight loss while our results indicate *Enterobacteriaceae* increased with weight gain.

CHAPTER 5. CONCLUSION

The WF, HF, and BW groups had the most weight gain after the eight week treatment period, while the LF treatment group had the lowest weight gain (Table 1). The greater the percentage of weight gain of our treatment groups fed a 45% fat diet compared to the LF group indicates that these mice in the current study were obese (Table 1). The average weekly food intake was significantly different among treatment groups ($p < 0.007$) (Table 1); however, we were unable to conclude weekly intake may have an effect on post treatment weight.

The post fecal samples with the most diversity of *Enterobacteriaceae* overall were observed to be in the WF and BW groups (Table 3). The groups with the highest prevalence of *Enterobacteriaceae* were WF, BW, and DF, which had the highest fermentable fiber among the treatment groups. The groups with the lowest prevalence of *Enterobacteriaceae* were FO and HF, which were the groups with the lowest fermentable fiber. Our results indicate that a potential relationship exists between high fermentable fiber diets and *Enterobacteriaceae* proliferation.

A potential relationship was identified between the prevalence of *Enterobacteriaceae* and post treatment weight in the WF group. The WF group was the treatment group with the most weight gain post treatment (Table 1) and was also with the highest prevalence of *Enterobacteriaceae* in post fecal samples among the treatment groups (Table 6), which may indicate a high prevalence of *Enterobacteriaceae* has an effect on weight gain (Table 1). Our results indicate there may be a relationship between an increase in *Enterobacteriaceae* prevalence and an increase in weight gain.

Application of Research

The results of this study may impact agriculture, microbiology, dietetics, health and wellness professionals, the food industry, and to an extent all human beings around the world

consuming food for energy. This research indicated that the supplementation of whole flaxseed into the diet is related to *Enterobacteriaceae* shed into the feces, which is related to weight gain. The overall interpretation of these findings may be important to increase our knowledge and understanding of how *Enterobacteriaceae* and potentially other microbial species may play a role in overweight and obesity (Ley et al, 2005; Santacruz et al, 2009). Health and wellness professionals, including dietitians, are interested in promoting information for achieving healthy diet and BMI to individuals and communities. Agriculture and food industry professionals may find the growth and use of flaxseed and buckwheat for human consumption to benefit health and wellness. Further research is encouraged to understand the role of bacteria in the GI tract related to obesity prevention and treatment.

Research Limitations

Limitations exist due to the unpredictable challenge of working with live animals as research subjects. All efforts have been made to correctly and adequately control the animal environment and experimental protocol; however, we cannot account for adverse events such as refusal to eat, signs of distress, infection, disease or illness, death, etc. The data of one mouse from the lean diet group have been omitted from the analysis due to displaying characteristics of illness and distress.

With consideration to the maturation of the mice over an eight week treatment period, there has not been a standard or completed method involving a suggested time frame for complete *Enterobacteriaceae* growth and turnover in the GI tract of this obesity animal model. Our approximation for an appropriate time frame to observe *Enterobacteriaceae* changes in the GI tract was eight weeks. This could be a sufficient amount of time for observable bacterial growth and turnover to occur and stabilize; however, further research with varying check points may be

beneficial to more deeply understand the microbiota and their overall environment and activity in the GI tract. More frequent time point observations, such as an additional four-week observational period, may be beneficial to observe changes occurring over time. Our methods also did not include PCR results, as other researchers in the literature have used.

This research also may not reliably reflect the same outcome in humans. We expect to see similar responses; however, the assumption cannot be made that the results will predict the same result in humans as it is with C57BL/6J male mice.

CHAPTER 6. FUTURE RESEARCH

As obesity becomes more prevalent in our society, scientific research will continue to develop a complete model to determine the causes of obesity, which will likely include the gut microbiota as a component (Harris et al., 2011). Recommendations for future research are based upon the limitation of this study, which includes research performed using human subjects rather than obesity animal models. A second recommendation would be to include identification of other species in the gut microbiota such as *Firmicutes*, *Bacteroides*, *Actinobacteria*, and others which may have an effect on overweight and obesity status. A third recommendation would be to use PCR and RT-qPCR methods performed by previous researchers Amar et al., 2011, Santacruz et al. 2009, Santacruz et al., 2010, and Karlsson et al., 2012. A fourth recommendation would be to complete blood sample analysis for the information regarding lipid profiles, and insulin resistance as performed by Xaio et al. (2013) and Park and Velasquez (2012). Application of the knowledge gained from additional research would be beneficial to multiple groups including those involved in agriculture, microbiology, dietetics, health and wellness professionals, the food industry, and for human beings for weight maintenance.

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APPENDIX A. IACUC APPROVAL OF PROTOCOL



November 26, 2012

Dr. Yeong Rhee
Health Nutrition & Exercise Science
EML

Re: IACUC Approval of Protocol, #A13019 "The role of flaxseed and buckwheat in control of obesity" Category C

Research Team: Y. Rhee, M. Pulkrabek, K. Hert, T. Heck

Approval Date: November 26, 2012
Current Approval Period: November 26, 2012 to November 26, 2015
Next Update Report Due: October 1, 2013

The referenced protocol (*received: November 1, 2012*) has been reviewed by the NDSU Institutional Animal Care and Use Committee and has IACUC approval as of the date indicated above.

The IACUC requests that you keep a copy of this protocol on file at the location or facility where the animals will be housed. During the course of this project, if you plan any significant changes in the protocol, a Change in Protocol Form outlining the proposed changes must be submitted to the IACUC, and IACUC approval granted, before implementation of the changes. A report and renewal of the project is also required on an annual basis. A reminder will be sent to you about a month before the report due date.

Please feel free to consult with NDSU's Attending Veterinarian, to ask questions or discuss any animal-related needs or concerns throughout the duration your project. The IACUC chair is also available if you have questions regarding animal welfare or university requirements.

NDSU has an Animal Welfare Assurance on file with the Public Health Service's Office of Laboratory Animal Welfare (OLAW). The assurance number is **A3244-01**, last renewed on May 25, 2010. NDSU is also registered with the U.S. Department of Agriculture as an Animal Research Facility under the registration number **45-R-002**.

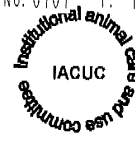
Thank you for your cooperation with NDSU IACUC procedures.

NDSU IACUC

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
NDSU Dept 4000 | PO Box 6050 | Fargo ND 58108-6050 | 701.231.8114 | Fax 701.231.8098 | ndsuiacuc@ndsu.edu
Shipping address: Research 1, 1735 NDSU Research Park Drive, Fargo, ND 58102

NDSU is an EO/AA university.

Sponsored Programs Administration
Office of the Vice President for Research, Creative Activities and Technology Transfer
1735 NDSU Research Park Drive, Dept. 4000, PO Box 6050
Fargo, ND 58108-6050, Phone (701) 231-8114, Fax (701) 231-8098



Office Use Only: IACUC #: <u>A13019</u>	Date Received: <u>11/1/2012</u>	Full Board _____	DR <u>X</u>
Approval Date: _____ Protocols, PTF associated: _____			
Approval Period: _____ - _____ Pilot Study: Yes <input type="checkbox"/> No <input type="checkbox"/>			

Animal Care and Use Application

For Institutional review only

Project Title

The role of flaxseed and buckwheat in control of obesity

Principal Investigator

Yeong Rhee

HNES

Department

Campus Address

351 EML

1-7476

Contact Phone

E-mail Address

yeong.rhee@ndsu.edu

List any additional contact information for animal-care issues (*optional*):

Anticipated duration of the study: Nov. 2012-Oct. 2013
(*Not to exceed three years*)

Yeong P. Rhee _____ Oct. 30, 2012 _____
Principal Investigator Date

Wanda Steward _____ 10/31/12 _____
Chair, Head, Director or Dean Date
(If PI is Dept. Chair/Head/Director, the Dean must sign)

The signature above certifies acknowledgment that this research is in keeping with the standards set by your department/unit, all NDSU policies and that facility, equipment and personnel are appropriately committed to this project

W. Rhee _____ 11/28/12 _____
IACUC Chair Date

Carefully review the application to ensure it is complete, contains sufficiently detailed responses to all questions and all attachments. **Incomplete applications will be returned or held until completed, without IACUC review or approval, potentially delaying the research.** Contact the IACUC office for questions or assistance at 231-8114.

1. Classification and funding:

- Category B (animals being bred, conditioned, or held for *but not yet used* in research or teaching)
 - Category C (animals used for teaching or research causing no or only momentary pain or distress (i.e. routine venipuncture), not requiring the use of anesthetics or pain-relieving drugs)
 - Category D (Animals used for teaching or research causing more than momentary pain or distress to the animals and for which appropriate anesthetic, analgesics, or tranquilizers will be used): *complete Search for Alternatives to Animal Use*
 - Category E (Animals used for research or teaching causing more than momentary pain or distress, without the use of pain relieving drugs (analgesics, anesthetics, or tranquilizers), or protocols where animals will be allowed to die as a planned result of the study, with no intervention (i.e. euthanasia)): *complete Search for Alternatives to Animal Use and Justification for Unrelieved Pain and Distress*
- Internally Funded: no grant or contracts associated
- Externally Funded. Source:
Associated NDSU Proposal Transmittal Form number or grant number:

2. Additional documentation. Check all that apply and provide appropriate form:

- Search for Alternatives to Animal Use (*classification D or E only*) – Appendix A
- Breeding – Appendix B
- Herd Management – Appendix C
- Justification for Unrelieved Pain and Distress – Appendix D
- Analgesics, Anesthetics, or Surgery – Appendix E
- Teaching or Classroom – Appendix F
- Wildlife and Free-Ranging Animals – Appendix G
 - Exempt from oversight (*Fill out and submit only the remainder of this form and the first page of the Wildlife and Free-Ranging Animals form*)

3. Will the focus and intent of the project be biomedical in nature?

- YES NO

4. Project activity. Describe the specific goals of this project clearly and concisely. Use language understandable to nonscientists and avoid acronyms.

- What is the purpose of the study?
- What potential benefits might be derived from the study?

The purpose of the study is to determine the role of flaxseed and buckwheat in obesity development and prevention.

The potential benefits of the study results are the contribution to a reduction of obesity prevalence using natural food products and create new knowledge to better understand the regulatory mechanisms of obesity.

Two graduate students and an undergraduate student will be trained in animal handling, feeding, and/or laboratory assays by the PI and University's Attending Veterinarian. The graduate students will be also responsible for obtaining weekly body weight of animals, measuring daily food consumption, and pair-feeding animals in Group 6.

Animals will be housed individually in plastic-bottom cages and kept in a temperature (22-25 C) and humidity (42-55%) controlled room with a 12-h light: dark cycle in the Animal Nutrition and Physiology Center at North Dakota State University. Fresh food and water will be provided daily. Animals will have access to food and water ad libitum. Animals will be weighed weekly and food consumption will be measured daily for pair-feeding and monitoring of food intake. Animals will be fed with experimental diets for 8 weeks. At week 0, 4, and 8, beddings will be removed from the cage for 12 hours and urine and fecal samples will be collected to measure gut microbiota activity such as urinary hippurate and fecal microflora analysis.

Following 8 weeks of experimental diet feeding, animals will be euthanized with CO2 gas, and then

exsanguinated by anterior cardiac puncture. Plasma samples will be separated and stored at -80 oC for future analysis. Liver, heart, skeletal muscle, intestine, spleen, pancreas, lung, and perirenal and epididymal adipose tissues will be collected, weighed, frozen in liquid nitrogen, and stored at -80 oC for future analysis. Obesity biomarkers such as glucose, insulin, lipids, leptin, adiponectin, TNF-alpha, IL-6, and /or C-reactive protein will be measured using plasma and tissue samples.

5. Procedures to be applied to animals.

Animals will be randomly assigned into one of six different treatment groups: Group 1 (45% Kcal fat, control); Group 2 (45% Kcal fat, 10% whole flaxseed); Group 3 (45% Kcal fat, ~6% Defatted flaxseed); Group 4 (45% Kcal fat, ~4.5% flaxseed oil); Group 5 (45% Kcal fat, 10% Buckwheat); and Group 6 (control diet, 12% Kcal fat, pair-fed to group 2). Group 6 will be pair-fed in which animals receive a 12% fat diet at restricted amounts reflecting feed intake of the 45% fat, whole flaxseed diet (group 2) on the previous day. Food consumption of mice in Group 2 will be measured daily, and the same amount of low fat control diet (12% Kcal fat without flaxseed supplementation) will be fed to mice in Group 6. Twelve mice will be assigned into each diet group. Three extra mice will be fed normal mouse chow, and these mice will be used for training of PI and students by Dr. Walden, University Attending Veterinarian.

6a. Animals to be used in this activity:

Species/strain* (include common name)	Sex	Age	Pain Category	Maximum Number of Animals used during project
C57BL/6J Mouse	Male	0-3 mont	C	75

*Strain is required if you have animals that are of specific genetic interest or are inbred, outbred, or transgenic.

6b. Source of animals. List vendor/breeder, stockyard, institution, other NDSU unit or herd, etc.

The Jackson Laboratory

6c. Justify the species and number of animals to be used, statistically when applicable.

Male C57BL/6J mice, 5-6 weeks old and ~20 g body weight, will be used for the proposed study. C57BL/6J mice are susceptible to diet-induced obesity [1, 2]. The selected animal develops obesity following a high fat diet (45%-60% kcals from fat) [3, 4].

From our pilot study [5] and subsequent power analysis, a minimum sample size at a power of 0.8 and a Type 1 error rate of 5% is n=12 in each group. A total of 72 mice will be fed experimental diets and additional 3 mice will be fed control diet. These 3 mice will be used for training of PI and students by Dr. Walden.

1. The Jackson Laboratory JAX® Mice Database. <http://jaxmice.jax.org/strain/000664.html>. Accessed October 26, 2012.

2. Surwit RS, Seldin MF, Kuhn CM, Cochrane C, Feinglos MN. Control of expression of insulin resistance and hyperglycemia by different genetic factors in diabetic C57BL/6J mice. *Diabetes*. 1991;40:82-7.

3. West D, Boozer C, Moody D, Atkinson R. Dietary obesity in nine inbred mouse strains. *Am J Physiol*. 1992;262:R1025-32.

4. Rossmeisl M, Rim JS, Koza R, Kozak L. Variation in type 2 diabetes-related traits in mouse strains susceptible to diet-induced obesity. *Diabetes*. 2003;52:1958-66.

5. Rhee Y, Brunt A. Flaxseed supplementation was effective in lowering serum glucose and triacylglycerol in glucose intolerant people. *JANA*. 2006;9:28-34.

7. Where will the animals be housed/located?

Animal Nutrition and Physiology Center

8. Where will procedures take place?

Animal Nutrition and Physiology Center

9. Will your project *require* any specialized project related housing or husbandry? (e.g. sterile cages, wire bottom cages, environmental enrichment devices, social isolation)

NO

10. What known or potential animal-related problems can be anticipated during this project?

Unexpected animal disease, infection, or death can be anticipated during this project. All necessary measures will be taken to keep the animals healthy throughout the study.

How will the above issues be addressed?

The PI/students will monitor animal health status closely, and notify Dr. Walden for possible treatment.

Under what circumstances will animals be removed from the project?

Animals with decreased food consumption for extended period of time, continuous weight loss, any significant signs of distress will be removed from the project

11. What will be the disposition of the animals used in this project?

- Euthanized in the lab, with or without further sampling postmortem; carcasses disposed of by incineration or other approved method
- Harvested in commercial facilities under state or federal inspection with carcasses entering the food chain
- Released back into the natural habitat
- Maintained under animal management conditions for future use in research
- Transferred to another project – list protocol number and investigator
- Other (describe)

12. **Euthanasia.** Describe euthanasia method(s) used as part of the study; also, *for all projects*, describe the method of euthanasia to be used in emergencies.

Animals will be euthanized in a CO2 gas chamber.

13. Is animal death (without euthanasia) a planned endpoint of the study?

- No Yes. If yes, provide a scientific justification.

14. Training documentation

Complete below for each person who will have any contact with live animals, *including the PI*, so that the IACUC can adequately assess qualifications and training for this protocol.

If the PI is not the person responsible for supervision of students or employees, provide the supervisor's name. *Supervisor training must be completed.*

Name	Position within University	Animal-related project duties	For IACUC office use only	
			Training Core module completed	Certification of Qualifications
Yeong Rhee	Associate Professor	Supervision of students' animal care, feeding, tissue collection	10/11/2012	
Margaret Pulkrabek	Graduate Student	Provide animal care: feeding, cage changes, weight measurement; assist in tissue collection	10/12/2012	
Kerrie Hert	Graduate Student	Provide animal care: feeding, cage changes, weight measurement; assist in tissue collection	11/6/2012	
Taylor Heck	Undergrad Student	Provide animal care: feeding, cage changes, weight measurement; assist in tissue collection	11/3/2012	

* To add personnel to after initial approval, complete the Addition of Personnel Form, sign and forward to the IACUC office.

15. Principal investigator certifications:

By signing the cover page of this protocol, I certify that:

- all the information provided is accurate to the best of my knowledge and I will adhere to the procedures described;
- all individuals listed as personnel on this project are trained and qualified for their specific duties involving animals under this proposal;
- all persons listed on this protocol have read the protocol or will be provided access to the complete protocol approved by the committee before engaging in any animal use related to this project;
- the activities described in this study do not *unnecessarily* duplicate previous experiments. If activities will duplicate previous experiments, I have included a written explanation and justification for the duplicative procedures.

and I agree to:

Protocol # A 13019

- obtain approval from the IACUC *in advance* of any changes in the project;
- notify the Attending Veterinarian and/or the IACUC of any unexpected study results that impact animal welfare;
- comply with guidelines in *The Care and Use of Vertebrate Animals at NDSU* and with NDSU Occupational Health and Safety Guidelines;
- be familiar with and comply with all pertinent institutional, state, and federal rules and policies;
- be responsible for the supervision and work of my staff;
- retain copies of this protocol and all correspondence associated with it for three years beyond the completion of the animal use;