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QUANTITATIVE ANALYSIS OF THE TOTAL BACTERIA, LACTOBACILLUS, AND BIFIDOBACTERIUM COLONIC MICROFLORA IN RATS FED CONVENTIONAL, PREBIOTIC, AND PROBIOTIC SOY DIETS

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by

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B.A., Southern Illinois University, 1987

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A Thesis
Submitted in Partial Fulfillment of the Requirements for the
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THESIS APPROVAL

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Approved by:

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AN ABSTRACT OF THE THESIS OF

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MAJOR PROFESSOR: Jeremy Davis, PhD

Research suggests that specific compositions of gut microbiota can directly affect energy harvesting and fat storage, which may indicate a potential role of intestinal bacteria in the regulation of body weight (i.e., obesity). The purpose of the current study was to determine if prebiotic- and probiotic-based diets modify gut microbiota in genetically obese rodents. For this, female Zucker diabetic fatty (ZDF) rats were assigned diets containing fructooligosaccharides (FOS), *Bifidobacterium* (BIF), or *Lactobacillus* (LAC) for three weeks. qPCR was then used to measure levels of colonic Bifidobacterium, Lactobacillus, and total bacteria. At termination, there was no significant difference in *Lactobacillus* levels between diets. However, there was significantly less *Bifidobacterium* in BIF vs. FOS or LAC-fed rats. The evidence in this study shows there were no significant differences in *Lactobacillus* levels between any of the feeding groups and the control group, supporting the conclusion that ingestion of any of the tested supplemented food does not statistically modulate *Lactobacillus* numbers in female ZDF rats. However, the rats from the *Bifidobacterium* and FOS feeding groups had significantly higher colonic *Bifidobacterium* levels than the control group from ingesting the supplemented food, indicating that the presence of the

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probiotic *Bifidobacterium animalis* subspecies *lactis* and the prebiotic FOS stimulated the growth of *Bifidobacterium*.

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DEDICATION

I would like to dedicate this thesis to the people who had the most to endure throughout the project, my family. My mother, Alma Osterholm, has helped me so much caring for and taxiing around her grandson so I could write or stay late after work to do research in the lab. She has been my rock and I surely could not have accomplished any of this without her. My father, Harvey Osterholm, I know is watching me from above saying, “It’s about time! But you did a good job, Honey.” My son, Adam Michael, has had to keep the noise level down, have less of my time and attention than he should have had, and generally put up with me constantly having my nose in my laptop, and I thank him for doing all those things in his sweet, laid-back manner and without a great deal of whining. He is the light of my life and the reason I want to better myself. And finally, my fiancé, Denny, has given up many fun weekends so I could work and has given me countless pep talks and atta-girls throughout the years. No one could ask for a better cheerleader and life partner. I love and appreciate all of you and all you do for me. I only hope I can make you proud in exchange for our lost quality time together.

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CHAPTER 1

INTRODUCTION

Obesity and Type 2 diabetes mellitus (T2DM) are at epidemic proportions, with obesity rates increasing markedly over the past three decades. According to the Centers for Disease Control Behavioral Risk Factor Surveillance System, there was no state with obesity prevalence ≥15% in 1990. However, in just over 20 years this rate increased to ≥30% in several states.[1]

Obesity results from an imbalance in energy intake and expenditure. Furthermore, obesity is linked to a variety of comorbidities, such as T2DM. Currently, >8% of Americans are diagnosed with T2DM, whereas an additional 27% are thought to be undiagnosed.[2] It is also estimated that approximately one in 400 people under 20 years of age in the U.S. have diabetes (type 1 or type 2).[2] The underlying factor contributing to increasing prevalence of T2DM is the corresponding rise in obesity rates.

Probiotics are classified as live nonpathogenic microorganisms, such as bacteria or yeast, which confer physiological health benefits through changes in the host gut microbiota.[3-5] Alternatively, prebiotics are selectively fermented ingredients (i.e., dietary fiber) that allow specific changes in the composition and/or activity of gut microbiota that also results in multiple health benefits for the host.[6] However, not all dietary fibers are be considered prebiotic, as some stimulate potentially harmful bacterial growth or metabolism, and thus are not selective to beneficial bacteria.[7] A plethora of research has investigated the positive effects of prebiotics and probiotics in humans. However, more recent

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data has indicated that changes in gut microbiota through consumption of probiotics and prebiotics may influence the progression of obesity and its related comorbidities through modifications to energy harvesting and fat storage in hosts.[8-11]

Statement of the problem. Preliminary research investigated the potential metabolic effects of prebiotic and probiotic based diets on genetically obese Zucker diabetic fatty (ZDF) rats.[12] However, this previous work did not measure the bacteria profiles in obese and diabetic rats.

Purposes of the study. The primary purpose of this study was to determine how consumption of prebiotics and probiotics modified gut microbiota in obese ZDF rats. Additionally, another purpose was to describe the bacterial profile in these animals associated with metabolic outcomes (i.e., hyperglycemia, adiposity, etc.).

Research questions. 1. Can we detect if probiotics or prebiotics made a significant difference in the number of microorganisms in the gut? 2. And, within those differences can we determine if we are selecting for either of the two genera we were measuring using q-PCR?

Statement of hypothesis. We hypothesized that the prebiotics and probiotics would increase intestinal microflora and that we would select for both genera. We also hypothesized that the bacterial changes would correlate with positive metabolic outcomes.

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CHAPTER 2

REVIEW OF THE LITERATURE

Obesity and T2DM are at epidemic proportions, with prevalence of each exhibiting a marked increase over the past three decades. Among the states that participated in the Centers for Disease Control Behavioral Risk Factor Surveillance System in 1990, 10 had prevalence of obesity less than 10% and no state had prevalence ≥15%. By 2000, no state had prevalence of obesity less than 10%, 23 states had a prevalence of 20–24%, and no state had a prevalence of ≥25%. However by 2010, no state had an obesity prevalence of less than 20% and 36 states had ≥25% with 12 of those having ≥30% prevalence (**Figure 1**). In 2012, no state had a prevalence of obesity less than 20%, nine states and the District of Columbia had a prevalence of between 20–24%, and 13 states had a prevalence ≥30%.[1] Obesity increases the risk of many health conditions, including coronary heart disease, stroke, hypertension, and cancers (i.e., endometrial, breast, and colon).[13]

Obesity results from increased intakes of energy-dense foods and decreased physical activity. However, when an entire population is exposed to the same nutritional stresses, certain individuals within that population do not experience similar weight gain and metabolic dysfunction. This suggests that there are additional mechanisms beyond energy intake and expenditure.[8]

Type 2 diabetes mellitus (T2DM) results in the body's inefficient use of insulin. In 2011, T2DM affected over eight percent of Americans with an additional 27% still undiagnosed.[14] About 3,600 people under 20 years of age in

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the U.S. are newly diagnosed with T2DM every year.[14] T2DM is largely caused from excess body weight and a lack of physical activity.[15]

Much research has been conducted over the years regarding the effects of prebiotics and probiotics in the diet. More recently, research has demonstrated that specific compositions of gut microbiota can directly affect energy harvesting and fat storage. These data indicate that prebiotics/probiotics may distinctly impact pathophysiology of obesity.[8-11]

Introduction to probiotics

A diverse and complex gut microbial ecosystem exists that is indispensable for the human host's health and wellbeing, even beyond the gastrointestinal tract. This environment is sterile in infancy, develops through childhood, matures in adulthood, and becomes more complex in old age. It is stable, but ever changing. Consequently, the "normal flora" is able to perform mechanistic roles that the human body cannot do itself.[16]

Modern definitions. The World Health Organization (WHO) defines probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host.[3] Another source affirms a probiotic to be a live microorganism that is administered to alter the intestinal microflora, thereby conferring a beneficial effect on the patient's health.[4] And another more recently states that probiotics are viable, nonpathogenic microorganisms (bacteria or yeast) that are able to reach the intestines in sufficient numbers to confer benefit to the host.[17] An integrated definition of probiotics is live, nonpathogenic microorganisms (bacteria or yeast) which when administered in adequate

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amounts and which reach the intestines in sufficient numbers confer a beneficial effect on the health of the host.

Microbial ecology of the GI tract

Each person has a unique and stable gut microbial environment, but community shifts do occur. Age, gender, diet, health, and medications (antibiotics, in particular) can cause variations in the general blueprint associated with the human GI tract.[18] About 3.3 million genes constitute the gut microbiome, about 150 times larger than the number of genes identified in the entire human genome. It is believed that up to 100 trillion microorganisms consisting of over 1000 species inhabit the individual adult intestine at any given time,[18,19] which is 10X the number of cells in the human body.[16] More recently, a study based on over 50,000 16S rRNA gene sequences distinguished about 1800 genera, 16,000 species, and over 36,000 strains of bacteria overall in the human intestine.[10]

Four bacterial groups comprise 98% of the total human gut microbiome: *Actinobacteria* (3%), *Proteobacteria* (8%), *Bacteroidetes* (23%), and *Firmicutes* (64%).[10] The latter group constitutes the largest percentage and contains within it two genera of interest to this research, *Bifidobacterium* and lactic acid bacteria of the genus *Lactobacillus*. They are considered “normal flora” of the gastrointestinal tract.

The acidic pH of the stomach prevents growth of most bacterial species. Only about 10^3 g^{-1} of intestinal contents survive this harsh environment and those that do are found predominantly on the walls of the stomach.[20,21] These are

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primarily Gram-positive facultatively anaerobic genera such as *Lactobacillus*,[21] though a few aerobic and Gram-negative bacteria can be found as well (**Figure 2**).

Bacterial concentration in the small intestine is typically between 10^4 and 10^8 g^{-1} contents and primarily consists of some facultative anaerobes and some strict anaerobes. The number of commensal bacteria in the small intestine is still limited by the low pH from stomach acid.[17] The pH of the duodenum is 6–6.5, but the area in and around the brush border can reach 7–8.[22] In the proximal small intestine (i.e., duodenum and jejunum) *Lactobacillus* and *Enterococcus* predominate with bacterial concentration ranges from 10^4 and 10^5 g^{-1}.[10,20] In the distal small intestine (i.e., ileum), the bacterial composition begins to resemble the large intestine, which includes the following genera: *Bacteroides, Clostridium, Eubacterium*, *Peptostreptococcus, Ruminococcus, Fusobacterium, Butyrovibrio, Enterobacterium, Lactobacillus,* and *Bifidobacterium.*[4] However relatively speaking, numbers of *Lactobacillus* drop [20] and gram-negative bacteria far outnumber gram-positive bacteria in the distal small intestine.[4] The pH in the jejunum and ileum is about 7.5.[22]

The microflora of the colon is one of the most densely populated microbial habitats known, around 10^{11} to 10^{12} g^{-1} contents [4,17,18,23] and is quite diverse consisting of at least 500 microbial species.[17] Bacteria make up about 60% of the mass of human fecal matter,[17] dominated by the genera *Bacteroides, Eubacterium, Fusobacterium, Butyrovibrio, Peptostretococcus, Clostridium, Fusobacterium, Ruminococcus*, *Lactobacillus,* and *Bifidobacterium.*[4,24] The large

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intestine contains much larger numbers of obligate anaerobes than facultative anaerobes.[25]

Species of the genera *Eubacterium, Lactobacillus*, and *Bifidobacterium* are typically used as probiotics in many dietary supplements and functional foods on the market today.[18,26] These probiotics function to out-compete pathogenic bacteria such as *Staphylococcus, Clostridium,* and *Pseudomonas*. Two of these probiotic bacteria, *Lactobacillus* and *Bifidobacterium,* are the most commonly studied probiotic genera in the literature, selected based on their consistently viable, safe, and metabolically active characteristics.

Health benefits of probiotic use

Modern humans are considerably less exposed to microbes than our ancient ancestors with the modern Western diet consisting of much less fiber, non-digestible carbohydrates, and whole plant foods (fruits, vegetables, whole grains) and much more protein, saturated fat, and refined sugar. Such health issues as allergic and inflammatory maladies, metabolic syndrome (including obesity, hypertension, dyslipidemia, glucose intolerance, and insulin resistance), cancer, diabetes, stroke, and cardiovascular disease [27-29] are prevalent in modern Western society. Many studies have focused on beneficial health effects attributed to gut microbes' symbiotic influence.

Improvement of bowel function. A notable benefit is the improvement of bowel habits. Irritable bowel syndrome (IBS),[28,30] diarrhea (particularly antibiotic-associated),[17,31] and ulcerative colitis [31,32] are among the most common gut problems studied with probiotic consumption.

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Good bowel function relies heavily upon short-chain fatty acid (SCFA) formation. From the breakdown of unabsorbed carbohydrates, several species of *Eubacterium* and other *Firmicutes* produce the SCFA butyrate, a major source of nutrition for colonocytes.[31,33] SCFAs have a positive effect on processes such as carcinogenesis and gene expression, energy metabolism, and cholesterol and lipid levels.[29] Diets deficient in dietary fiber have decreased production/ concentration of fecal SCFAs[34]; however, it was observed that humans who consumed a restricted carbohydrate diet with probiotics maintained SCFA production.[35] Furthermore, after the administration of probiotic supplements for six months, the microbiota associated with IBS patients changed toward that of IBS-free patients.[30]

The symptoms of acute diarrhea have been improved with probiotics by 21% and that of antibiotic-associated diarrhea improved by 52%.[5] *Lactobacillus* have been particularly effective for improving the symptoms off chronic diarrhea.[31] In eight of ten randomized controlled trials, it was reported that a significant number of participants experienced improvement from antibiotic-associated diarrhea using the probiotic yeast *Saccharomyces boulardii.*[36] Many GI disorders are treated with broad-spectrum antibiotics that actually exacerbate the disease with osmotic diarrhea and diarrhea associated with opportunistic and/or antibiotic resistant pathogenic bacteria, such as *Clostridium difficile* and *C. perfringes, Salmonella, Staphylococcus aureus,* or *Proteus*. As such, probiotic therapy can be used to quickly re-establish the normal flora and provide effective competition against pathogens.[10,37-39]

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In patients with ulcerative colitis (UC), probiotics have been shown to relieve symptoms.[31] Fecal samples from UC patients can contain very high concentrations of lactate.[33] Some bacteria, including species of *Eubacterium* and *Bifidobacterium*, are able to ferment lactate to butyrate and this could explain how those without UC remove lactate from the colon.[33] However, there are other colonic bacteria that also can produce butyrate, so the evidence for probiotics is still unclear in UC cases.

Immune system benefits. The gut-associated lymphoid tissue (GALT) is a substantial component of the body's immune system. Research has discovered that probiotics modulate the intestinal immune response[18] and help to combat allergies.[35,40] For example, *Lactobacillus salivarius* and other probiotics positively influence Natural Killer cells and monocytes. These leukocytes affect both the innate and specific immune responses.[31,35]

Certain strains of *Lactobacillus* clearly play a role in the development and function of dendritic cells [special cells for presenting antigens to T helper cells (cells which signal immune responses)].[41,42] The majority of dendritic cells in the GI tract are immature, and are subject to maturity based on their environment.[41] Mature dendritic cells produce the cytokine IL-12, which is important in differentiation of T_H1 subset helper T cells.[41,42]

Reduced intestinal permeability. The main entry points for most pathogenic bacteria are on luminal mucous membrane surfaces.[20] Secretory Immunoglobulin A (sIgA) binds antigens on pathogens, entrapping them within a hydrophilic shell and preventing them from attaching to mucosal cells and

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colonizing the colon.[17,42] Probiotics have been shown to raise sIgA levels in the luminal mucous layer.[17]

Once in the gastrointestinal tract, lactic acid bacteria produce bacteriocins, bacteriocidal (killing), or bacteriostatic (growth inhibiting) peptides.[5,43] Bacteriocins destroy gram-negative bacteria by penetrating the inner membrane or interrupting cell wall synthesis.[5] Bacteriocins are promising as future antimicrobial agents because thus far there have been no side effects or resistance reported with their use.[43] Additionally, due to the fact that they are proteins, which are easily degraded.[43]

Probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* produce SCFAs by fermenting 80–90% of the carbohydrates that the human host cannot digest (i.e., dietary fiber).[18,31] The production of both SCFAs and high levels of lactic acid by lactic acid bacteria creates conditions detrimental to the pH-sensitive cytoplasmic membrane of pathogenic bacteria (e.g., *C. perfringens*) and this causes the cells to lyse.[10,20] SCFAs are beneficial to the host metabolism by increasing intestinal motility, absorption, defecation frequency, lipid and carbohydrate metabolism, mucus production, and blood flow to the large intestine.[35,44,45]

Obesity link. A more recent hypothesis relates to the connection of obesity with the microbial composition of the gut. In mice, a mutation in the leptin (*ob*) gene causes deficiency of the adipose-regulating hormone leptin and is linked with early-onset obesity.[46] The gut microbial community of genetically obese (*ob/ob*) mice contains 50% fewer *Bacteroidetes* species and 50% more

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Firmicutes than their lean wild-type (+/+) littermates.[9,11] Furthermore, the intestinal microbiota of obese mice is less diversified and contained a greater number of methanogens (*Archaea* that produce methane).[10]

When feces of genetically obese mice was measured by bomb calorimetry, results disclosed substantially less energy than their wild-type siblings (**Figure 3a**).[9] The proposed mechanism is that members of the *Firmicutes* convert more dietary fiber to short chain fatty acids, hence the host animal gets more fat from the same amount of food.[10] Within two weeks, the germ-free mice experienced a significant increase in adiposity despite equal or decreased food quantity, indicating that obesity is transmissible (**Figure 3b**).[9,11] The conclusion is that the microbiota regulate the host's harvest of energy and organic nutrients from the diet.

In a study with human subjects, a similar *Bacteroidetes/Firmicutes* relationship was found regardless which diet the subjects were on (fat-restricted vs. carbohydrate-restricted). Interestingly, this correlation remained consistent following significant weight loss (**Figure 4**).[11] Moreover, there was a significant correlation between the increase of *Bacteroidetes* and weight loss percentage (**Figure 5**).[11]

As mentioned previously, methanogens are more abundant in the gut of *ob/ob* mice. Methanogens remove the products of fermentation from the gut, in particular, acetate, hydrogen (H_2), and carbon dioxide (CO_2). The removal of H_2 from the gut in the reduction of CO_2 to methane makes the energetics of fermentation more favorable. This in turn, makes available additional nutrients for

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the host to absorb, contributing to obesity.[10] This has imminent therapeutic implications for the treatment of obesity.[9]

More benefits of SCFAs. In addition to providing energy for colonocytes and lowering the pH of the colon to maintain the integrity of the mucosa, there are several other positive functions from the breakdown of nondigestible carbohydrates to SCFAs by probiotic bacteria. A summary of some of the benefits of probiotics, many well studied as well as others in earlier research stages, are listed in Table 1.

Prebiotics

Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon.[7] This definition has more recently been updated to selectively fermented ingredients that allow specific changes. Both in the composition and/or activity in the gastrointestinal microbiota confer benefits upon host well-being and health.[6] Consequently, not all dietary fibers can be considered prebiotic, as some dietary fibers stimulate potentially harmful bacterial growth and/or metabolism, and so are not selective to beneficial bacteria.[7]

Humans lack the opacity to digest dietary fiber. As such, enteric microflora to catabolize them.[47] When lactic acid bacteria (primarily *Bifidobacterium*) ferment fiber, the pH of the large intestine is reduced.[20] Therefore, the growth of bacteria that are already resident in the colon can be accelerated with the use of prebiotics, which provides an advantage over simple probiotic usage.[6,48]

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Interestingly, prebiotics possess differing fermentation profiles, so some metabolites can be converted or used as substrates for other prebiotics — this is called “metabolic cross-feeding”.[49] Typically, the competition will positively affect those groups that are considered to be healthy for the host[47] and negatively affect unfavorable bacteria.[49-51] A prime example is by the anaerobic gut bacteria converting lactate produced by *Bifidobacterium* into butyrate and other SCFAs.[48]

A requirement to be considered a prebiotic is that it must reach the colon as a fermentable substrate, meaning it has to be at least partially unhydrolyzed and unabsorbed in the small intestine.[50-52] Prebiotics do not replace the normal flora but spur growth of the lactic acid bacteria such as *Bifidobacterium* and *Lactobacillus*, or activate them metabolically, or both. Thus, the colonic microbial composition is shifted by prebiotic consumption.[47,51]

Specific prebiotics. Dietary fiber such as cellulose, lactosucrose, polydextrose, indigestible dextrin, soy polysaccharide, rice bran, and chitosan can alter the microbiota.[4] In this country, the fructans are the most common prebiotic additive used in food.[28] This group includes oligosaccharides such as galactooligosaccharides, lactulose,[18] and short-chain fructooligosaccharides (synonymous with oligofructose) found in garlic, artichokes, onions, bananas, tomato, leeks, and wheat.[28] Additionally, they are manufactured from sucrose or extracted from chicory.[28,52,53] Xylooligosaccharides, isomaltooligosaccharides, and soybean oligosaccharides have all been touted as having prebiotic qualities, however more research is needed to bump them from their classification of tentative prebiotics to established prebiotics.[52]

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Synbiotics

Synbiotics are probiotics in the form of live microorganisms and certain prebiotics available in the same product concurrently.[18,44,50] Synbiotics increase the likelihood that the probiotics survive and thrive since a preferred substrate, the prebiotic, is easily accessible.[51]

The different segments of the intestine have disparate substrate concentrations and pH levels. A study of several combinations of probiotics and prebiotics found an optimum combination with a strain of *Lactobacillus acidophilus* with mannitol, fructooligosaccharides, and inulin.[44] A list of examples of common probiotics, prebiotics, and synbiotics can be found in Table 2.

There are so many probiotic products on the market, it is difficult for consumers to know what to purchase. There are many varieties with regards to formulations, stability, and quality control, as well as the problem of matching the appropriate probiotic with the disease or condition experienced by the patient, much less when prebiotics are added to the mix (synbiotics).

Recommended dosages of prebiotics vary depending on the type of prebiotics consumed. Differing ranges of dosages are the result of the variable fermentation characteristics of the prebiotics.[28] Package labels with terms such as starch, corn starch, modified food starch, and maltodextrin are indicators of resistant fiber, but not all are resistant to digestion, so the savvy consumer still must consult the actual fiber content on the nutritional label.[28]

Typical carbohydrates of the human diet consist of resistant starch, non-starch polysaccharides, such as cellulose, hemicellulose, inulin, and pectin, non-

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absorbable sugars and sugar alcohols, and chitin and amino sugars — none of which are well fermented by *Lactobacillus*.[20] They may instead rely on the hydrolytic activity of bacteria such as *Bifidobacterium* and *Bacteroides* for their carbohydrates.[20] However, they can ferment prebiotic carbohydrates such as galactosaccharides, fructooligosaccharides, raffinose, stachyose, lactitol, and palatinose.[54]

Preliminary studies

The original feeding study on which this thesis is based was conducted by Michele Martin under the direction of Dr. D. Allan Higginbotham with the purpose of determining anti-obesity and anti-diabetic effects of prebiotics and probiotics added to soy protein diets in the female Zucker diabetic fatty or ZDF-Lepr^{fa}/Crl (ZDF) rat model.[12] The ZDF emanates from the inbreeding of hyperglycemic Zucker obese rats.

Male ZDF rats have an *fa* gene mutation in which the leptin receptor protein does not interact with leptin (the cytokine product of the *ob* gene which increases energy expenditure and decreases food intake, thus lowering body weight).[55,56] This results in constant messages of hunger being sent out by the hypothalamus, and continuous eating ensues.[57]

When put on a regular (Purina 5008, 16.7% kcal fat) diet the obese female ZDF rats will gain weight but remain euglycemic and will not develop diabetes. [58] Female ZDF rats were used in the preliminary study because when subjected to a high fat diet (27% fat was used in the preliminary feeding study), they develop Type 2 Diabetes Mellitus.[12]

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The hypothesis was that the prebiotics and probiotics would affect intestinal microflora and maximize the anti-obesity and anti-diabetic effect of the soy protein. The research questions were whether FOS, *B. animalis* subsp. *lactis*, or *L. acidophilus* in a soy-based diet decrease body weight and fat gain, and improve glycemic control in a preclinical model of T2DM. These strains were chosen as they have been well studied for their biological action and potential applications for commercial probiotic supplements.

A soy-based protein diet was chosen based on evidence that it could improve glycemic control.[12] Intestinal microflora may modify undigested soy protein components (possibly the isoflavones), and could be a method to improve glycemic control and prevent weight gain. Soy isoflavones affect glucose and triglyceride metabolism, which in turn, affect insulin levels.[12] Soy polysaccharides can improve glucose tolerance by reducing glucose and triacylglycerol concentrations.[12]

A 60-day study using rabbits as their animal model found that daily ingestion of a probiotic (*Enterococcus faecium* and *Lactobacillus helveticus*) soy product resulted in significant increases in fecal *Lactobacillus* and *Bifidobacterium* species counts compared to the control group. The unfermented soy food did not increase those bacterial populations. The experimental group also was found to have a reduced risk of cardiovascular disease.[59]

Many different animal models have been used to study probiotics, including human. Important data can be gleaned using tissues from an animal host that would not be accessible from human models. By using rats in this

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experiment, the entire colon could be extracted in order to take into account intestinal adhesion across the epithelium common with some strains of probiotic bacteria. Other tissues were taken from these experimental rats for the preliminary study.[12] Even if the dosages/specific organisms used are not realistic/appropriate for human comparisons, we can potentially obtain relevant results from animal experiments suitable for advancing our understanding of the use and activity of probiotics in humans.

This study aimed to determine changes in *Lactobacillus*, *Bifidobacterium*, and total bacteria in the colon and feces of rats fed conventional, prebiotic, and probiotic soy-based diets using these obese diabetic rats. The research questions are: can we detect if probiotics or prebiotics made a significant difference in the number of microorganisms in the gut; and, within those differences, can we determine if we are selected for either of the two study genera using q-PCR?

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CHAPTER 3

MATERIALS AND METHODS

Animals and Diet. Twenty-four female obese Zucker Diabetic Fatty rats (ZDF-Lepr^{fa}/Crl; Charles River Laboratories, Raleigh) were block randomized by body weight to control or experimental diets (n=6) (**Table 3**). All diets were isocaloric and isonitrogenous with 50% of total kcal from fat. Experimental diets were supplemented with 2.5% of fructooligosaccharide (FOS), *Bifidobacterium animalis* (BIF), or *Lactobacillus acidophilus* (LAC). Animals were housed in the Southern Illinois University Carbondale Vivarium under the supervision of the Institutional Animal Care and Use Committee (IACUC). Rooms were temperature controlled and light regulated, and rats were housed in individual wire cages. Food was measured daily and rats were weighed three times per week.[12]

All diets contained 27% fat. The control soy diet was comprised of 42.9% carbohydrate and 17.9% starch while the FOS, *Bifidobacterium,* and *Lactobacillus* soy diets all consisted of 40.4% carbohydrate, 25% sucrose, 15.4% starch, and 2.5% functional (see Table 3 for complete composition of experimental diets).

Sample Collection. The feeding study was conducted for three weeks until rats exhibited severe hypoglycemia with lesions. Rats were euthanized and tissue samples collected. A colon sample was also removed from each rat, placed in a sterile conical tube, and immediately immersed in liquid nitrogen until samples were placed in an ultra-cold (-80°C) freezer.

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Bacterial acquisition, growth conditions, and isolation. Bacterial control strains were obtained from the SIUC Department of Microbiology's stock culture collection and were grown under semi-aerobic conditions overnight in 10 ml screw-capped tubes partially filled with Bacto Tryptic Soy (TS) broth at 37°C. These strains were used as controls for primer optimization (**Table 4**).

Two strains intended for use in this research, *Lactobacillus acidophilus* (NRRL B-4495) and *Bifidobacterium animalis* (NRRL B-41405) were obtained from the culture collection of Dr. Alejandro P. Rooney at the U.S. Department of Agriculture's National Center for Agricultural Utilization Research in Peoria, Illinois. Due to problems growing a sterile *B. animalis* culture, *B. infantis* was later acquired from Chr. Hansen, Inc. (I-Powder-50; Milwaukee). The taxonomic descriptions of the genera *Lactobacillus* and *Bifidobacterium* are shown in **Appendix A**.

Probiotic strains were grown aerobically overnight in MRS broth in partially filled 10 ml screw-capped tubes or on agar plates in BBL Gas-Pak™ jars (Becton Dickinson, Sparks, MD) at 37˚C. The MRS medium (containing trypticase soy powder, dextrose, beef brain heart infusion, peptone, sodium acetate, yeast extract, sodium citrate tribasic dihydrate, sodium phosphate dibasic anhydrous, polysorbate (Tween) 80, magnesium sulfate heptahydrate, manganese sulfate tetrahydrate, thiosulfate, L-cysteine hydrochloride, MOPS, and cobalamin) was adjusted to pH 6.5 ± 0.2 at room temperature, brought to volume, and gently heated to boiling. Medium was then added to loosely capped 10 ml screw-

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capped tubes and autoclaved at 121°C for 20 min. For culture plates, Bacto™ agar was added to the medium prior to the boiling step.

Quantitative PCR. To optimize primers, relative quantification of bacterial DNA in samples was determined using quantitative real-time PCR (qPCR), which is 10–100 times more sensitive than the plate-count method.[60] Pure cultures of all bacterial control and study strains were harvested from a centrifuged bacterial pellet from 10 ml of culture and subsequently isolated using the E.Z.N.A.™ Bacterial DNA Isolation Kit (D3350, Omega Bio-tek, Inc., Norcross, GE) according to manufacturer's instructions.

Comparison tests were performed to evaluate primers sets for optimum determination of relative *Bifidobacterium*, *Lactobacillus*, and total bacteria concentration in colon samples (**Appendix B**). The *16S gBifid*, *16S Lact*, and *16s p338fGC/P518R* primer sets were used for measurement of *Bifidobacterium*, *Lactobacillus*, and total bacteria, respectively. All primer sets tested were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and are listed in **Table 5**. Primer specificity was confirmed by real-time qPCR with genomic DNA from overnight cultures. No cross-reactivity was found with any of the non-target species tested.

Quantification of *Bifidobacterium*, *Lactobacillus,* and total bacteria was achieved with real-time qPCR with ribosomal DNA-targeted genus-specific primers using the CFX96™ Real-Time PCR Detection System (Bio-Rad Life Science Research, Hercules, CA). Each plate experiment was replicated three times and each reaction was carried out in triplicate in a volume of 15 µL using

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96-well optical-grade plates (MLL9601, Bio-Rad Life Science Research). Each plate contained three no-template controls. The qPCR reactions were designed as follows: 95ºC for 10 min (1X), 95ºC for 10 sec (40X), 57ºC for 30 sec (1X), 72ºC for 30 sec (1X), and 95ºC for 10 sec (1X). The C_t values were averaged for each animal and primer set. Relative concentrations were calculated using the $2^{-\Delta\Delta}C_T$ method.[61] FastStart DNA Master SYBR Green (Roche, Indianapolis) was used for all qPCR reactions.

Statistical Analysis*.* Data were analyzed with SPSS 20.0.0 (SPSS Inc., Chicago, IL) and tested for normal distribution using the Shapiro-Wilk test. Non-parametric data were transformed and one-way analysis of variance (ANOVA) test was performed. If ANOVA data were significant ($p<.05$), post hoc comparisons were then made between individual groups using Tukey's test. Mean differences were considered significant at $p<.05$.

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CHAPTER 4

RESULTS

This study was conducted to determine how the consumption of prebiotics and probiotics modified gut microbiota in obese ZDF rats and to describe the bacterial profile in these animals associated with metabolic outcomes (i.e., hyperglycemia, adiposity, etc.). The experiments were designed to detect if soy food supplemented with 2.5% fructooligosaccharides (FOS), *Bifidobacterium animalis* (BIF), or *Lactobacillus acidophilus* (LAC) made a significant difference in the number of microorganisms found in the gut; and, within those differences, if it could be determined that either *Lactobacillus* or *Bifidobacterium* were selected for.

Lactobacillus levels in obese female ZDF rats. Following three weeks on experimental diets (**Table 3**), there were no significant differences in *Lactobacillus* levels detected between diet groups F = 0.087, p=0.97; **Figure 6**). To determine whether DNA levels of *Lactobacillus* ($^{\Delta}C_T$) were associated with reported metabolic outcomes in female ZDF rats (**Appendix C**), Pearson's correlation coefficient (*r*) was used to assess the relationship with blood glucose (**Figure 7A**) and body weight (**Figure 7B**). There was no significant correlation observed between *Lactobacillus* levels and blood glucose (*r*=0.10, p=0.96) or body weight (*r* = -0.24, p = 0.27).

Bifidobacterium levels in obese female ZDF rats. Unlike *Lactobacillus*, there was a significant difference in *Bifidobacterium* content at termination (F=9.46, p<0.001; **Figure 8**). More specifically, *Bifidobacterium* was lower in

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female obese ZDF rats fed BIF compared to LAC or FOS ($p<0.05$) (**Figure 8**). There was also a significantly greater *Bifidobacterium* in FOS vs. CON fed rats (data not shown; $p<0.05$). Pearson's correlation coefficient (r) was then used to determine if these differences in *Bifidobacterium* levels were associated with changes in blood glucose (**Figure 9A**) or body weight (**Figure 9B**). There was no significant correlation observed between *Bifidobacterium* levels and blood glucose ($r=0.017$, $p=0.87$) or body weight ($r=-0.04$, $p=0.95$).

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CHAPTER 5

DISCUSSION & CONCLUSIONS

The purpose of this project was to quantitatively compare the probiotic and total gut bacteria levels of female Zucker diabetic fatty (ZDF) rats whose soy-based diet was supplemented with prebiotic FOS, probiotic *B. animalis*, or probiotic *L. acidophilus*. The evidence in this study shows there were no significant differences in *Lactobacillus* levels between any of the feeding groups and the control group, supporting the conclusion that ingestion of any of the tested supplemented food does not statistically modulate *Lactobacillus* numbers in female ZDF rats. However, the rats from the *Bifidobacterium* and FOS feeding groups had significantly higher colonic *Bifidobacterium* levels than the control group from ingesting the supplemented food, indicating that the presence of the probiotic *Bifidobacterium animalis* subspecies *lactis* and the prebiotic FOS stimulated the growth of *Bifidobacterium*.

FOS supplementation of 0.25% FOS significantly stimulated intestinal numbers of *Lactobacillus* over their control group and significantly lowered the populations of the harmful bacteria *E. coli* and *C. perfringens* in a study with broiler chickens.[62] However, the 0.50% FOS feeding group gained less body weight, had less *Lactobacillus* measured in their intestines, and more *E. coli* and *C. perfringens* than the 0.25% FOS group, indicating that it was excessive to supplement at the 0.50% FOS level.[62] This may hold some significance in that the preliminary study used 2.5% FOS supplement and the results of this study increased but showed no significance.

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In most probiotic studies *Lactobacillus* counts are increased after probiotic consumption. However, one probiotic study involving elderly volunteers reported a decrease in the genus *Lactobacillus* bacteria in fecal counts after feeding a probiotic cocktail of various *Lactobacillus* and *Bifidobacterium* species.[63] The researchers speculated the differences in results may have been caused by differing isolation and identification techniques used.[63] Another study involving healthy, elderly volunteers yielded significantly higher numbers of bacteria from the genus *Bifidobacterium* during the two-week synbiotic (containing *Bifidobacterium* capsules and ingested oligofructose) feeding period and during the three-week post-feeding period.[64] The relative levels of colonic *Bifidobacterium* of rats in the FOS feeding group differed from that of the control and *Bifidobacterium* feeding groups, probably reflecting the prebiotic effect of FOS, as it is known to stimulate *Bifidobacterium* numbers when administered as a dietary supplement.[65] *B. animalis* was detected in the bowel of rats administered food supplemented with the prebiotic inulin compared to the control group in another study.[66] And in another, the mean level of *Bifidobacte4rium* was between 2.2 and 3.5 times higher in short chain (sc)FOS-fed piglets than in control animals.[53]

The results of this study are similar to the studies discussed above in that *Lactobacillus* counts did not alter significantly, but *Bifidobacterium* counts did increase significantly. They indicate that an interesting addition to our study would have been to add two synbiotic feeding groups, *Bifidobacterium*/FOS and *Lactobacillus*/FOS. Results from previous studies have shown that an easily

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accessed, preferred substrate increases the viability of the probiotic.[51] In order for all the rats to begin the experiment with a “sterile” gut, they could have been given a round of antibiotics beforehand. Also, although *Lactobacillus* wis not significantly higher in this study, it is trending higher. Therefore, it is possible that with a higher *n*, the result may have become significant.

The hypothesis of the preliminary study was that the prebiotics or probiotics added to the soy protein could have some effect on the intestinal microflora. In the *Bifidobacterium*-fed group, lipid levels were higher than any of the other feeding groups. It was concluded that the higher concentration of probiotics likely produced SCFA and amino acids which were absorbed in the colon and subsequently increased body lipids.[12] It is likely that the *Firmicutes* in the gut are responsible for this conversion.[10]

Using male rats in the study would provide another statistical group. Further, use of a rat species that was not obese or prone to diabetes would likely have resulted in a longer, more complete study of the effects of the different diet groups since these rats developed diabetes so quickly and severely that the study had to be stopped. In addition, it is possible that the wire-floor cages could have been stressful for the animals as opposed to litter on a smooth cage floor, causing the rats to eat more than they normally would have. This could alter the ratios of the gut bacteria genera, thereby negatively skewing the results.[62]

A stool kit was used in DNA recovery from the colon samples for this study. This method/kit may select for certain bacteria because not all bacteria lyse equally well.[67] Gram positive bacteria cell walls can be harder to penetrate

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than those of Gram negative bacteria.[29,68] PCR reaction efficiencies may select for bacteria with lower GC ratios than say, *Bifidobacterium*, due to various melting and renaturation efficiencies.[29] Even slight variations in DNA sequences can cause non-detection of certain bacteria.[68]

Copy number variation is an application that requires accurate, absolute quantification. This now can be accomplished using a recently developed technology called droplet digital PCR that was not available at the time of data collection for this research. This technology allows only about one target molecule per reaction, and then hundreds or thousands of these reactions are run in parallel.[69] These reactions are not quantitative, but can determine the proportion that contains template for the target in question, and precisely determine copy number in the original sample.

Recommendations*.* There has been a vast amount of research done in the field of prebiotics and probiotics demonstrating health improvements, but there is a lack of cause and effect answers. Future research needs to link prebiotic and/or probiotic modifications with precise physiological actions leading to specific health benefits. The colonic measurements performed in this study cannot be indicative of prebiotic and probiotic feeding effects on the small intestinal ecosystem as a major target of viable probiotic strains. Furthermore, the small intestine is the most important site of energy absorption. Therefore, the results of this study cannot lead to any suppositions toward understanding the relationship between obesity and the microbiome. After recent discoveries of this

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relationship, modern research likely will focus heavily on this aspect of prebiotic and probiotic supplementation.

Metagenomics approaches will provide valuable genetic information regarding gastrointestinal bacteria. For example, the recent Human Microbiome Project directed by the National Institutes of Health (NIH) is sequencing and analyzing the genomes of about 600 GI bacteria. This study will examine the roles of microbial communities in the gut as well as bacteria from other human body sites, how they all interact with each other, and the relationship between disease and changes in the human microbiome.[70-72] This will give us a global view of the potential beneficial effects of probiotic and other commensal intestinal bacteria.

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TABLES

Table 1. *Summary of some benefits of probiotics administration to humans*

Decrease incidence of dental caries [28]
Attenuate lactose intolerance symptoms [28]
Alleviates mucosal inflammation due to *H. pylori* infection [73]
Reduce intestinal pathogens:
reduce intestinal permeability by modifying epithelial barrier function [17]
inhibit the growth and survival of pathogenic bacteria [4,10,17,31]
interrupt bound pathogenic bacteria [17]
promote defense barrier functions on the gut epithelial cells [74]
interrupt DNA, RNA, or protein synthesis/structure, or penetrate the inner membrane of gram-positive pathogens [17]
produce high levels of lactic acid and SCFAs to the detriment of pH-sensitive pathogens [10,20]
increase intestinal motility, absorption, defecation frequency, lipid and carbohydrate metabolism, mucus production, and blood flow to the large intestine from production of SCFAs [35,44,45]
modify epithelial barrier function by reducing intestinal permeability [17]
Improve bowel function:
produce digestive enzymes [51]
reduce symptoms of irritable bowel syndrome (IBS) [28,30]
shorten duration of infant infectious diarrhea [28]
reduce symptoms of diarrhea (particularly antibiotic-associated) [17,31]
reduce symptoms of ulcerative colitis [31,32]
provide energy for colonocytes [31,33]
lower the pH of the colon to maintain integrity of the mucosa [41,75]
Immune system benefits:
increase intestinal immune response [18]
combat allergies [35,40,76]
improve atopic dermatitis in children 2 years and over [77]
affect development and function of dendritic cells [41]
raise sIgA secretion levels into the luminal mucous layer [17]
repress rotaviruses [51]
Reduce respiratory infections [28]
Prevent urinary tract and vaginal infections [78]
Treat infections during pregnancy [78]
Retard carcinogenesis process [44]
Lower hypertension [79]
Obesity link [9,11]

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Table 2. *Examples of common probiotics, prebiotics, and synbiotics*[1]

Probiotics
Lactobacillus
L. acidophilus
L. casei
L. delbrueckii subsp. bulgaricus
L. reuteri
L. brevis
L. cellobiosus
L. curvatus
L. fermentum
L. plantarum
Gram-positive cocci
Lactococcus lactis subsp. *cremoris*
Streptococcus salivarius subsp. *thermophilus*
Enterococcus faecium
Streptococcus diacetylactis
Streptococcus intermedius
Bifidobacterium
B. bifidum
B. adolescentis
B. animalis
B. infantis
B. longum
B. thermophilum
Prebiotics
FOS (e.g., oligofructose and neosugar)
Inulin
GOS
Lactulose
Lactitol
Synbiotics
Bifidobacterium + FOS
Lactobacillus + lactitol
Bifidobacterium + GOS

[1] Some still under evaluation.
FOS, fructooligosaccharides; GOS, galactooligosaccharides. [51]

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Table 3. *Composition of experimental diets*[1]

Ingredient[2]	CON	FOS[3]	*BIF*[4]	*LAC*[5]
Soy[6]	200	200	200	200
Sucrose	250	250	250	250
Starch	179	154	154	154
Functional	0	25	25	25
Soybean oil	70	70	70	70
Lard	200	200	200	200
Fiber	50	50	50	50
Vitamins	10	10	10	10
Minerals	35	35	35	35
Cysteine	3	3	3	3
Choline	2.5	2.5	2.5	2.5
BTHQ	0.014	0.014	0.014	0.014

[1]Energy Density: 4.85 kcal/g^{-1} (Protein 17%, Carbohydrate 33%, and Fat 50% of total kcal)

[2]ICN Biomedicals, Costa Mesa, CA

[3]FOS diet contains 2.5% fructooligosaccharide

[4]*Bifidobacterium animalis* subspecies *lactis* (1.0 x 10^{10} cfu g^{-1}, Lyoferm, Inc., Indianapolis)

[5]*Lactobacillus acidophilus* (1.0 x 10^{9} cfu g^{-1}, Lyoferm, Inc., Indianapolis)

[6]Supro® Soy Protein Isolate, Solae LLC, St. Louis, MO

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Table 4. *Bacterial strains used as controls during the primer optimization*

Bacteria	Gram-stain
Lactobacillus acidophilus	+
Bifidobacterium infantis	+
Bacillus cereus	+
Staphylococcus aureus	+
Enterococcus faecalis	+
Enterobacter aerogenes	–
Proteus vulgaris	–
Salmonella typhimurium	–
Escherichia coli	–

Table 5. *qPCR primers tested for Bifidobacterium, Lactobacillus, and total bacteria concentration*

Primer	Target Genera/Group	Annealing Temp (ºC)	Sequence 5'→3'
Lact-F	*Lactobacillus (Leuconostoc, Pediococcus, Aerococcus,* & *Weissella*, but not *Enterococcus/ Streptococcus*	52.8	CACCGCTACACATGGAG
Lact-R		52.7	AGCAGTAGGGAATCTTCCA
g-Bifid-F	*Bifidobacterium*	53.7	GGTGTTCTTCCCGATATCTACA
g-Bifid-R		55.6	CTCCTGGAAACGGGTGG
recA-F	*Bifidobacterium*	57.8	CGTYTCBCAGCCGGAYAAC
recA-R		58.5	CCARVGCRCCGGTCATC
P338FGC	Domain *Bacteria*	61.3	ACTCCTACGGGAGGCAGCAG
P518R		58.7	ATTACCGCGGCTGCTGG
8F	Domain *Bacteria*	54.3	AGAGTTTGATCMTGGCTCAG
529R		65.1	ACCGCGGCKGCTGG

FIGURES

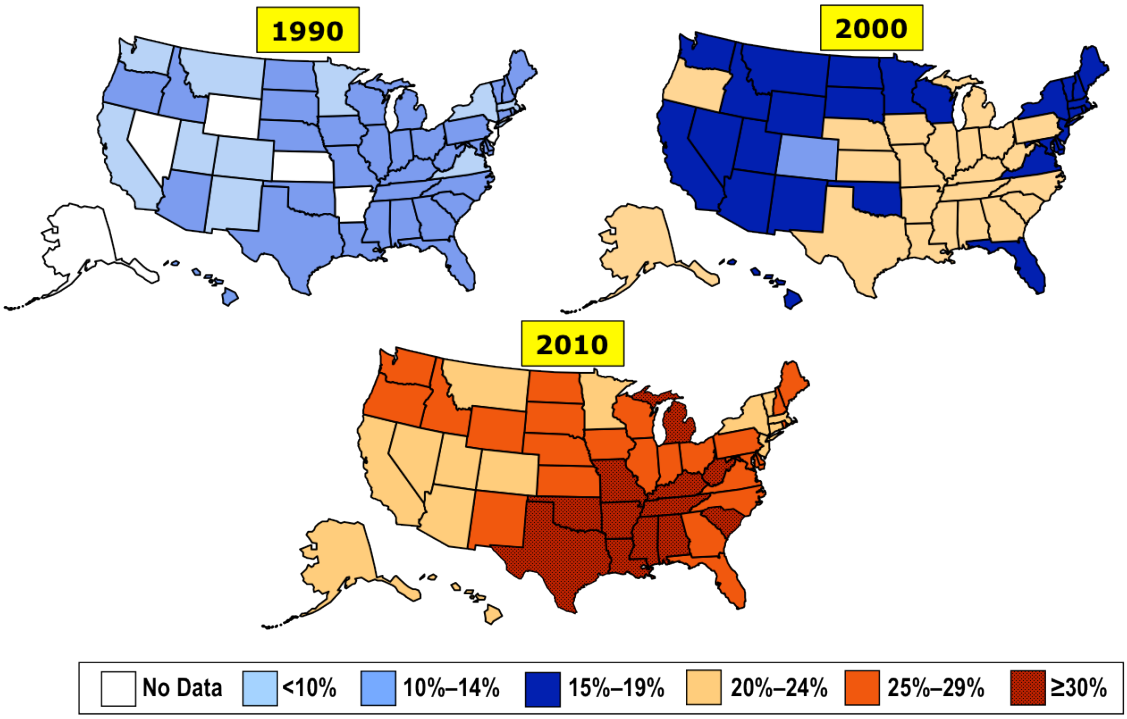

Figure 1. Obesity trends among U.S. adults. The CDC's Behavioral Risk Factor Surveillance System reports indicate obesity trends according to BMI calculated from self-reported telephone interviews with U.S. adults in 1990, 2000, and 2010. Obesity is indicated by a BMI of ≥30 (e.g., about 30 lbs. overweight for 5'4" person) [1]. In 1990, no state had a prevalence of obesity ≥15%. However, by 2012, several states had a prevalence ≥30%.

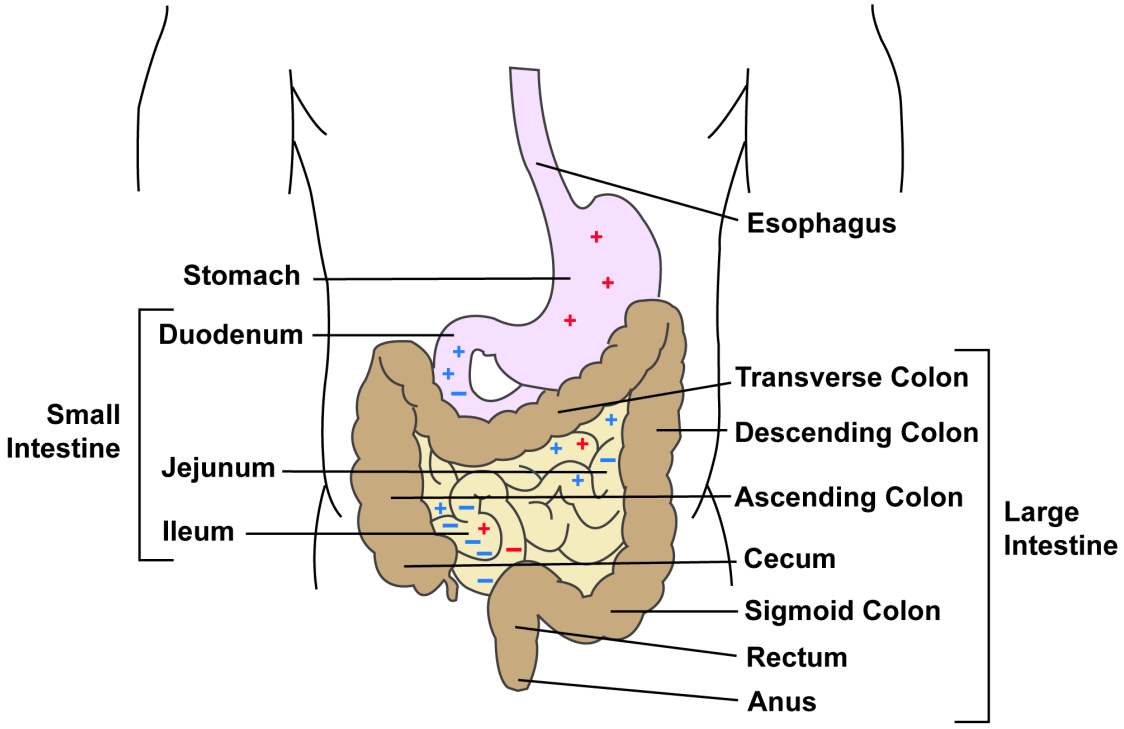

Figure 2. The gastrointestinal tract, illustrating bacterial quantities (each symbol ≈ 10-fold cells. Aerobic (red) vs. anaerobic (blue), and Gram-negative (–) vs. Gram positive (+).[4,21]

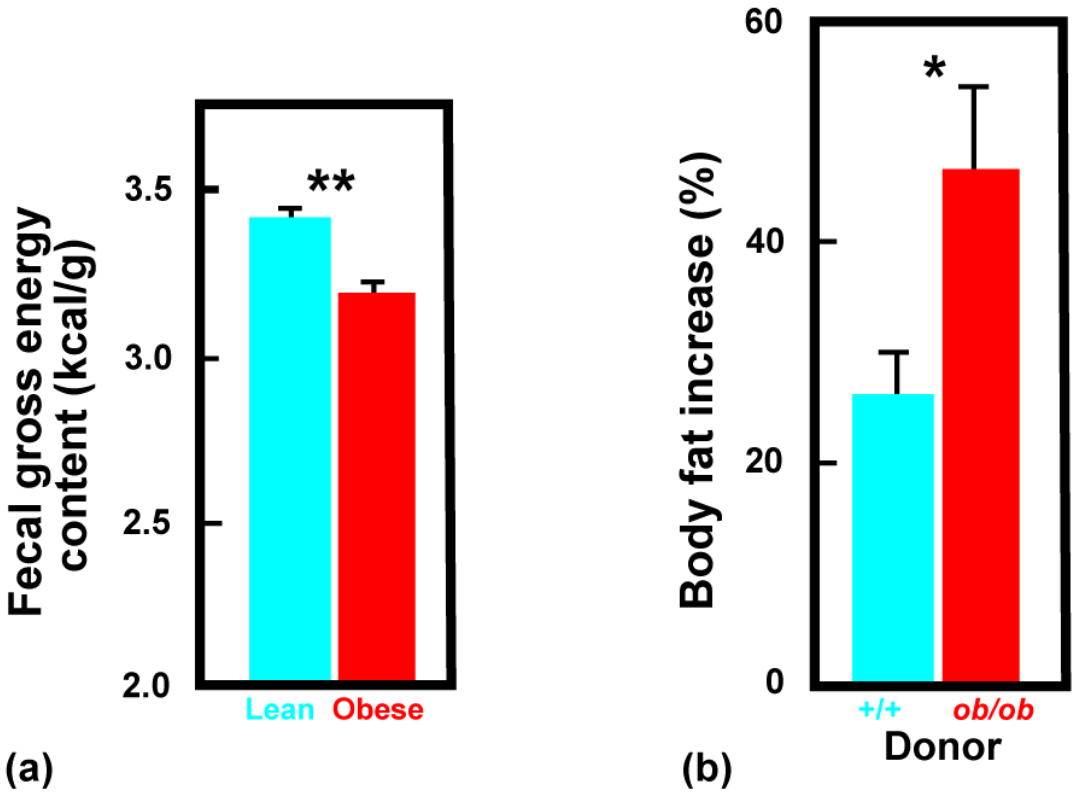

Figure 3. Microbiota transplantation. These experiments demonstrate that *ob/ob* microbiota harvest more dietary energy than their wild-type littermates. (a) Fecal gross energy content of lean (*+/+, ob/+*; n=9) vs. obese (*ob/ob*; n=13) C57BL/6J mice demonstrates that obese mice have significantly less energy that remains in their feces than lean wild-type mice. (b) Germ-free wild-type mice colonized with microbiota from the cecum of obese (*ob/ob*) donor mice displayed a significantly higher percentage increase in body fat percentage over wild-type mice colonized from lean (+/+) donors. Figure adapted Turnbaugh et al. (2006) [9].

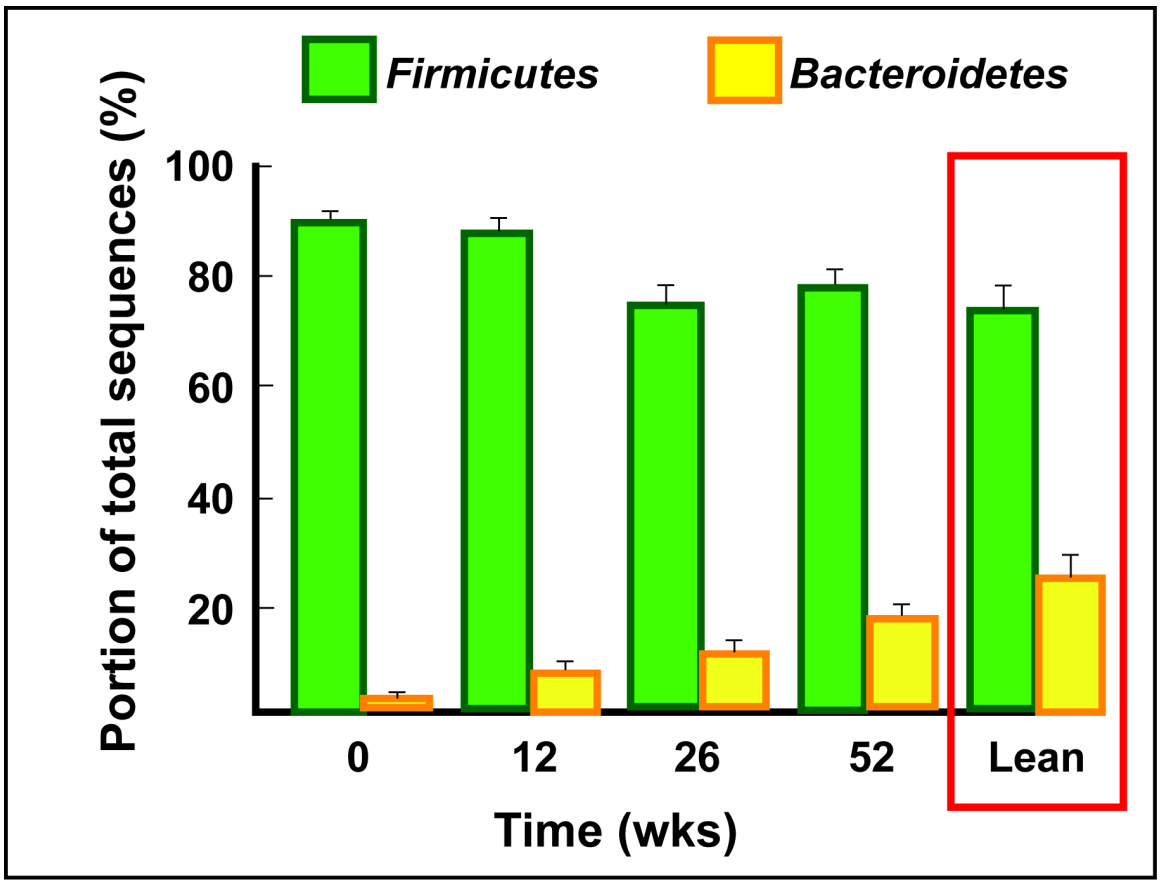

Figure 4. Relative abundance of *Firmicutes* and *Bacteroidetes*. In a one-year human study, the relative abundance of *Firmicutes* and *Bacteroidetes*. Sample average values at each time point (n = 11 or 12/time point). Lean controls averaged one year apart. Figure adapted Ley et al. (2006)[11].

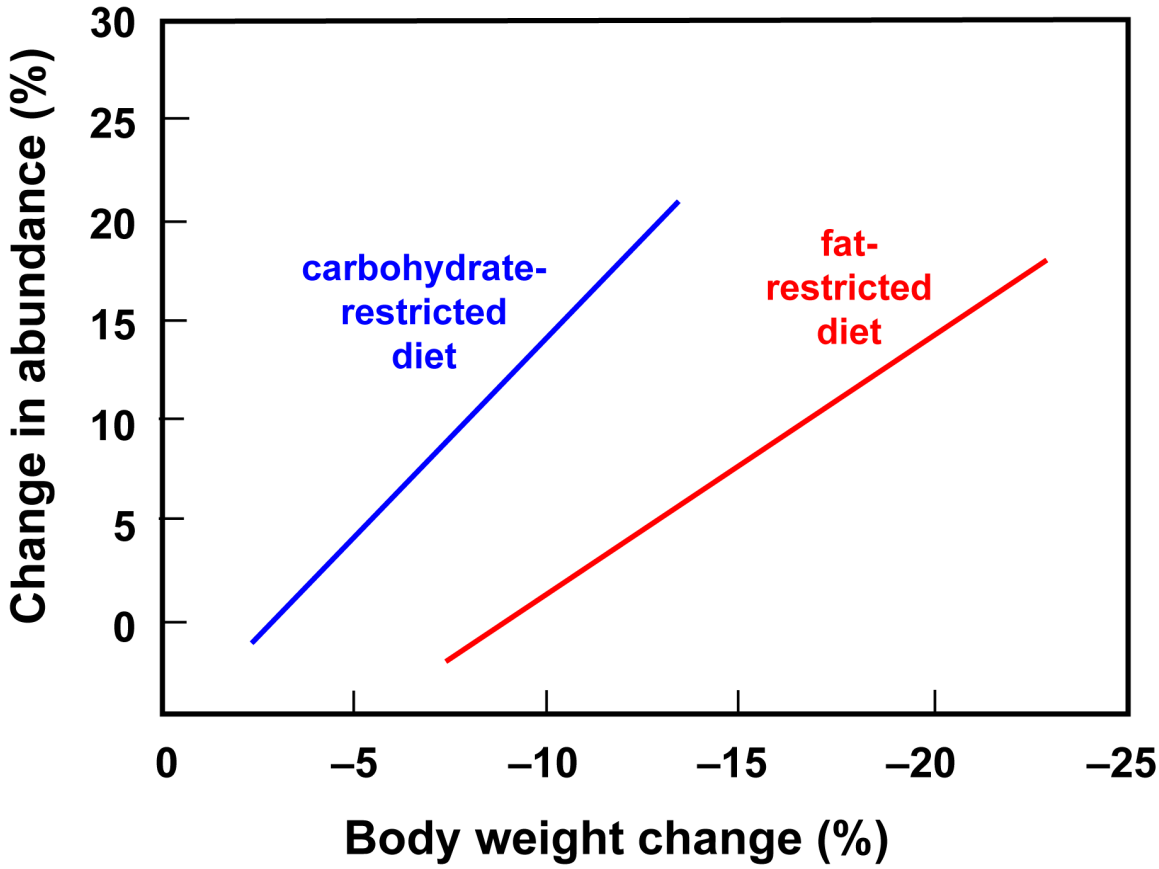

Figure 5. *Bacteroidetes* relative change in abundance. The *Bacteroidetes* group increased in abundance according to percentage of body weight lost in a study of a carbohydrate-restricted diet group with subjects who experienced greater than 2% body weight loss and subjects from a fat-restricted diet group who lost greater than 6% body weight. Figure adapted Ley et al. (2006)[11].

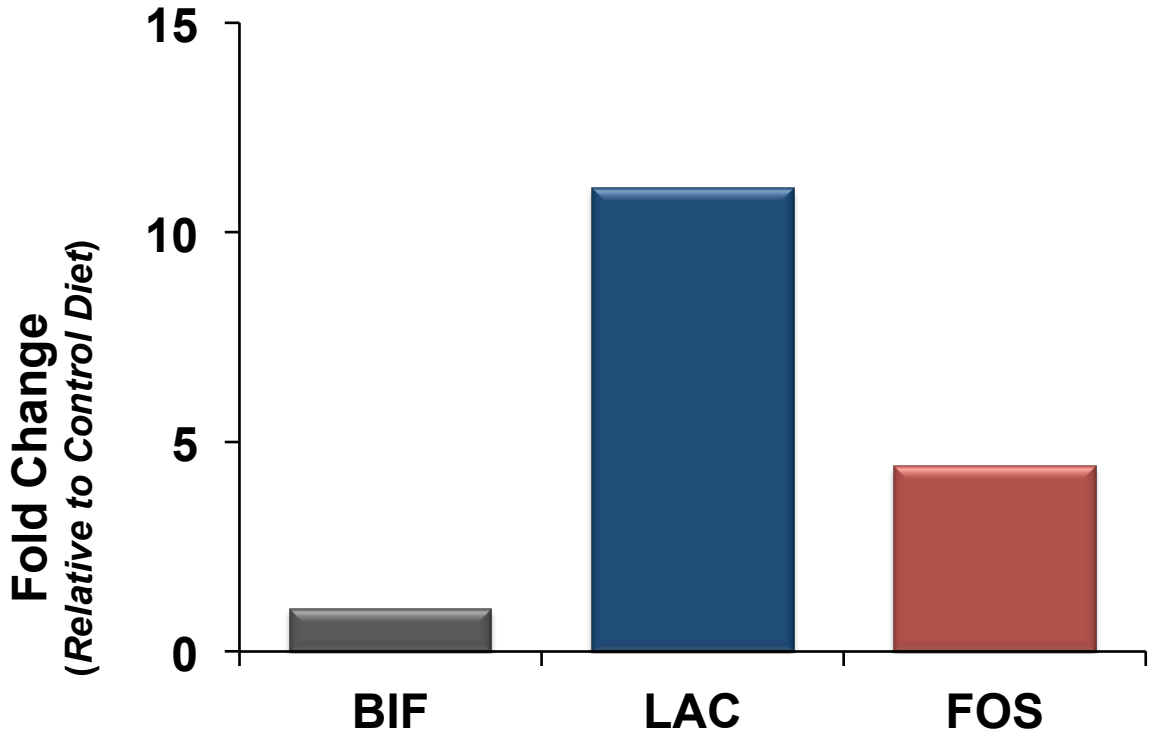

Figure 6. The abundance levels of *Lactobacillus* in DNA extracted from colon of female ZDF rats. Data values represent mean fold change calculated using the $2^{-\Delta\Delta C_T}$ method[61]. Data were analyzed using $^{\Delta}C_t$ values for each sample (C_t *Lactobacillus* $_n$ – C_t Total Bacteria $_n$), n = 24. The data were tested for normality (Kolmogorov-Smirnov, p=0.13 and Shapiro-Wilk, p=0.18) and analyzed using a one-way analysis of variance (ANOVA). There was no significant difference in abundance levels of *Lactobacillus* (F=0.087, p=0.97).

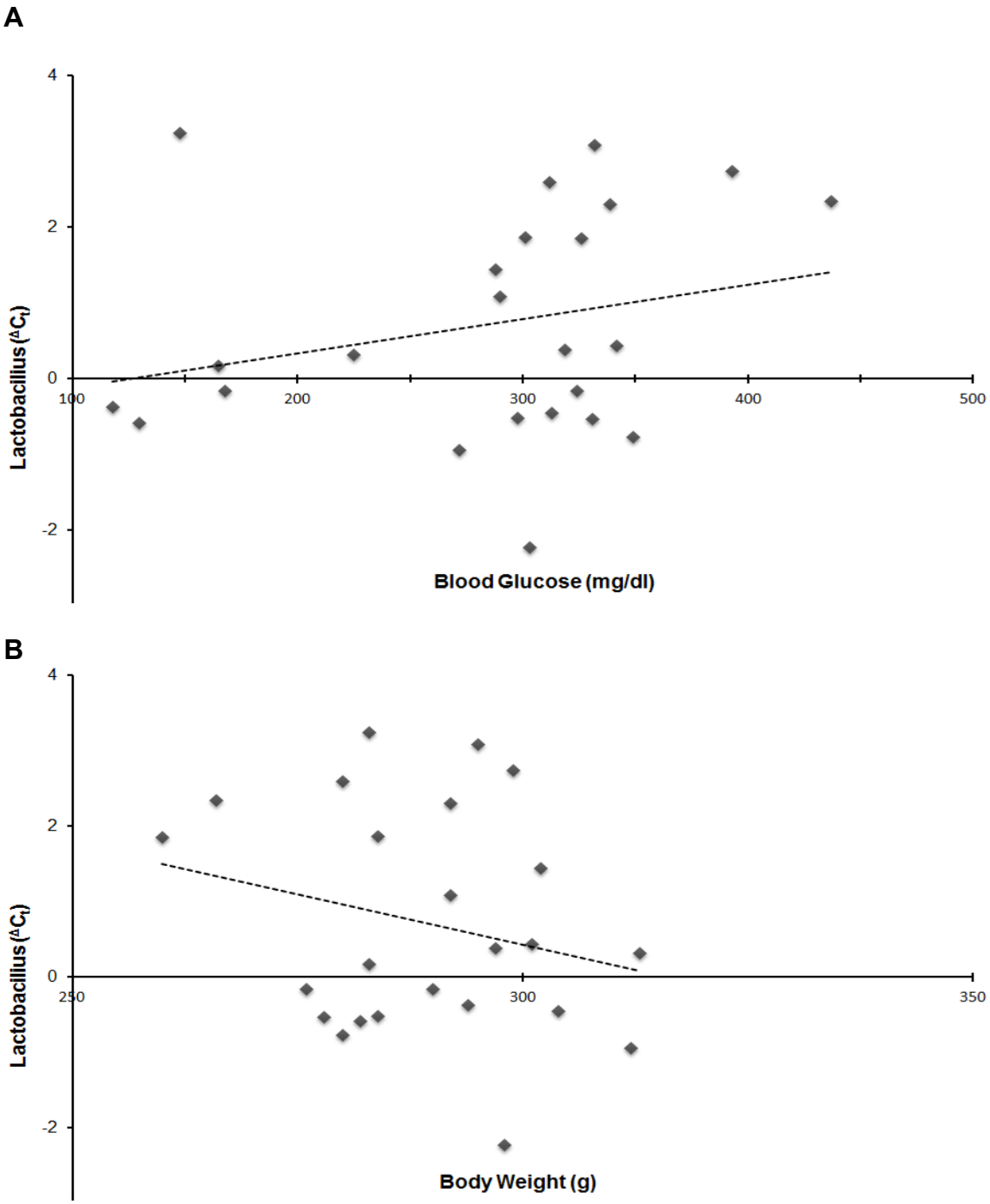

Figure 7. Relationship between *Lactobacillus* levels in colon and blood glucose and body weight in female ZDF rats. Scatter plot analysis of relative *Lactobacillus* levels (fold change) and (**A**) fasting blood glucose concentration (mg/dl), and (**B**) body weight (grams). Pearson's correlation coefficients (*r*) were *r*=0.10 (p=0.96) and *r*=-0.24 (p=0.27) for glucose and body weight, respectively.

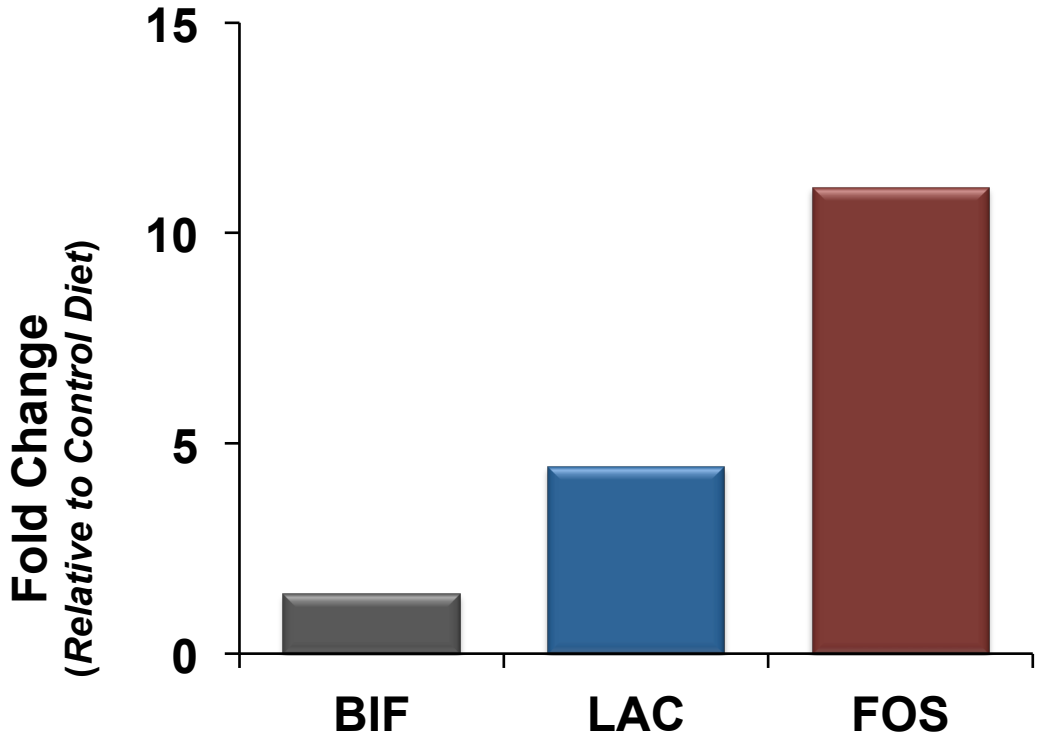

Figure 8. The abundance levels of *Bifidobacterium* in DNA extracted from colon of female ZDF rats. Data values represent mean fold change calculated using the $2^{-\Delta\Delta CT}$ method [61]. Data were analyzed using $^{\Delta}C_T$ values for each sample (C_T *Bifidobacterium* $_n$ – C_T Total Bacteria $_n$), *n* = 24. The data were tested for normality (Kolmogorov-Smirnov, p=0.13 and Shapiro-Wilk, p=0.18) and analyzed using a one-way analysis of variance (ANOVA). There was a significant difference in abundance levels of *Bifidobacterium* among groups (F= 9.46, p<0.001). Multiple comparisons were then made using Tukey's HSD test. Significant differences (at p<0.05) between means were indicated by different letters.

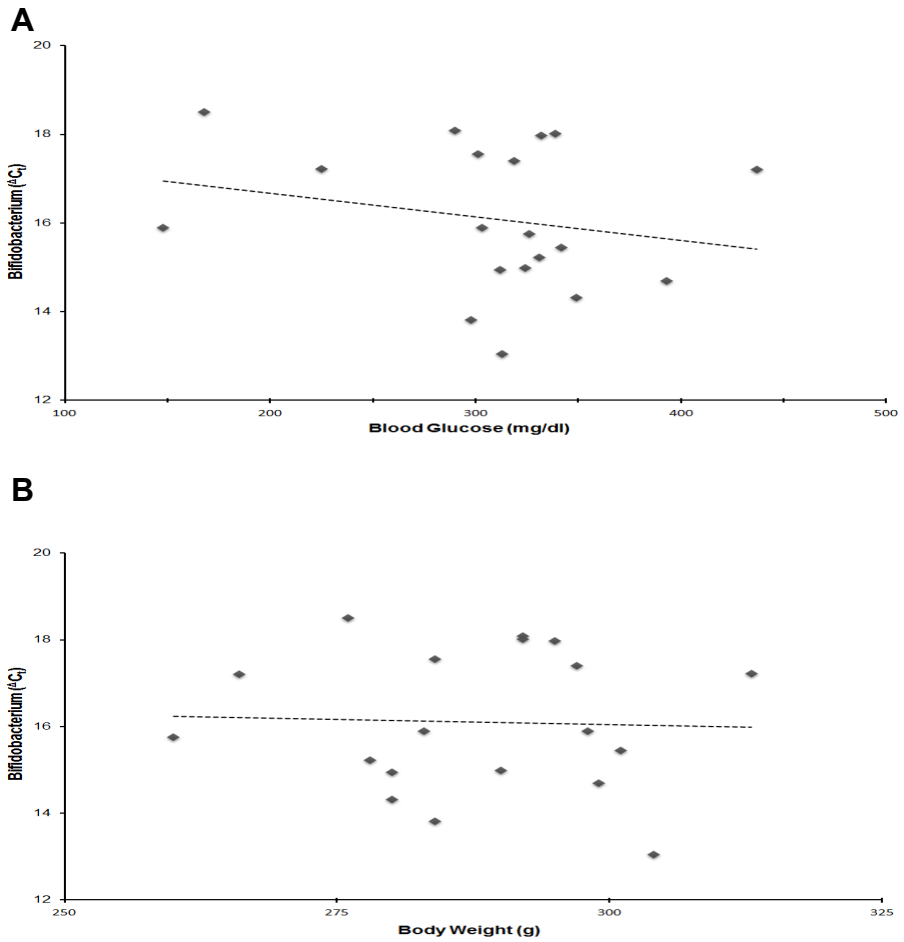

Figure 9. Relationship between *Bifidobacterium* levels in colon and blood glucose and body weight in female ZDF rats. Scatter plots analysis of relative *Bifidobacterium* levels (fold change) and (**A**) fasting blood glucose concentration (mg/dl), and (**B**) body weight (grams). Pearson's correlation coefficients were 0.017 (p=0.87) and -0.04 (p=0.95) for glucose and body weight, respectively.

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APPENDICES

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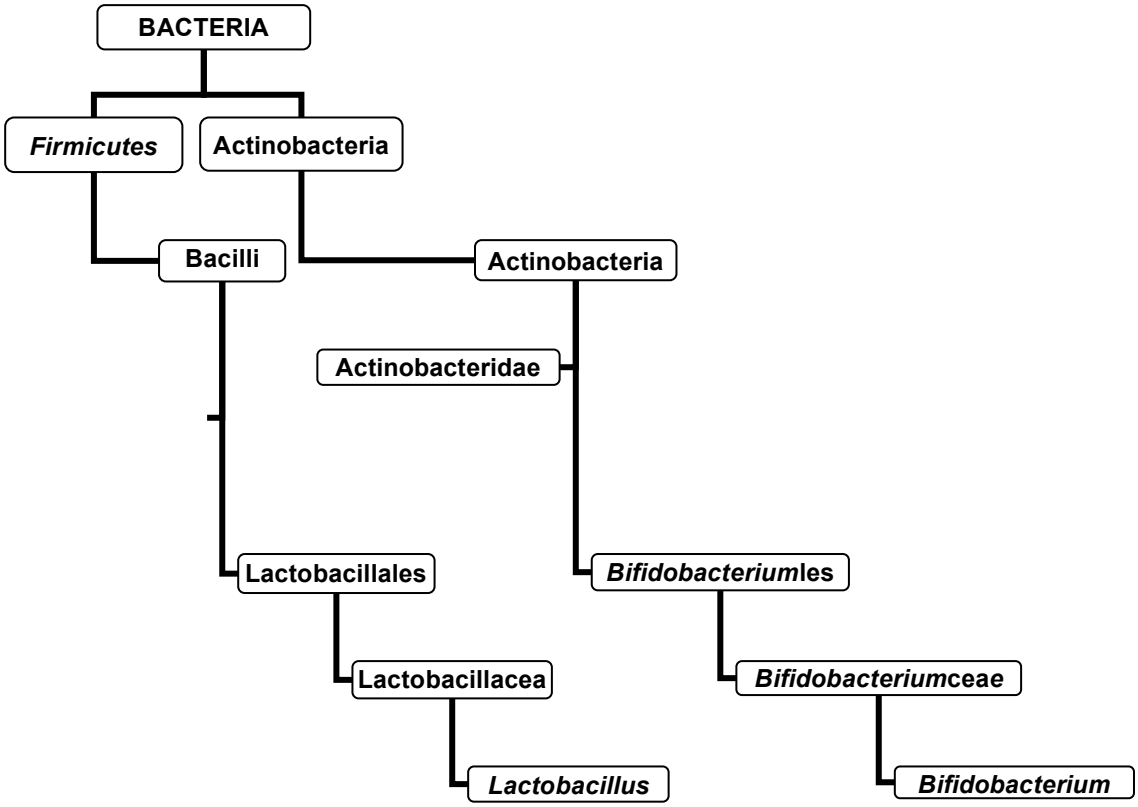
APPENDIX A

Taxonomic Description of Genera *Lactobacillus* and *Bifidobacterium*



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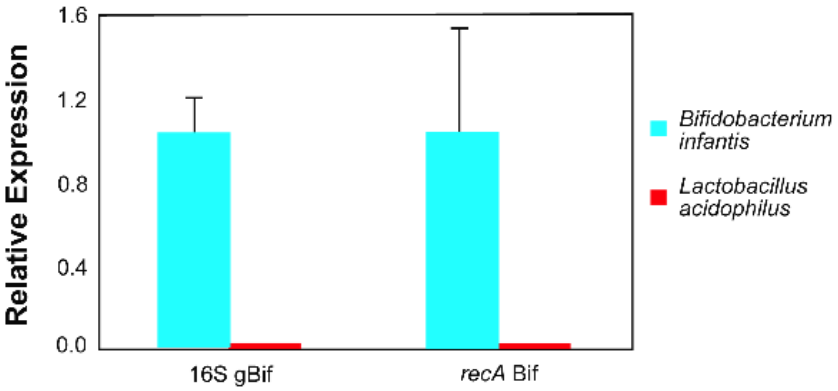
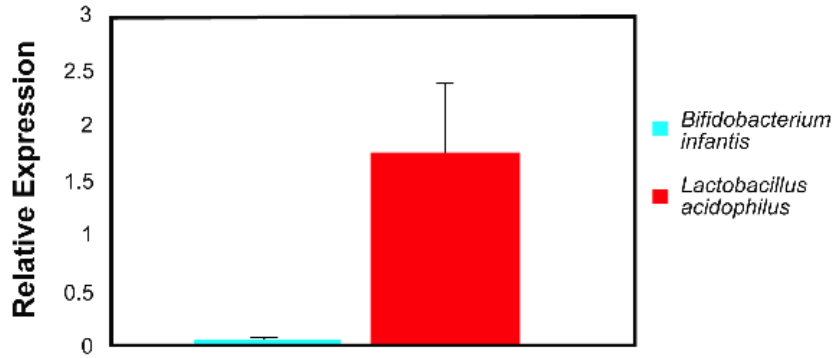
APPENDIX B

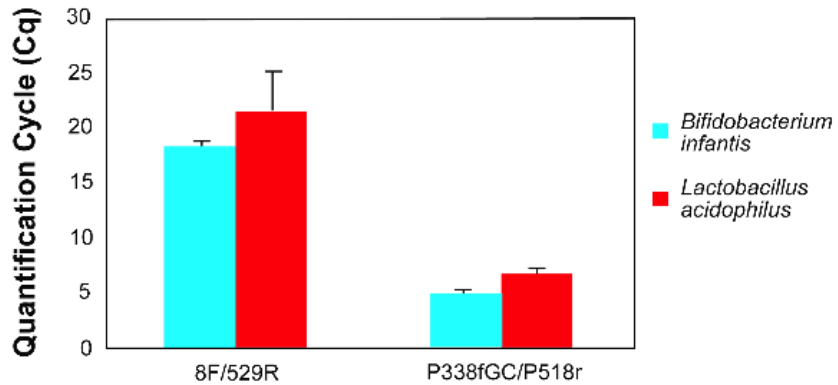
qPCR primer tests for *Bifidobacterium*, *Lactobacillus*, and total bacteria



Bifidobacterium. Graphical representation of real-time qPCR results comparing primer sets to detect genus *Bifidobacterium*, tested by using DNA of control bacteria, *Lactobacillus infantis*, and *Bifidobacterium animalis* subspecies *lactis*.



Lactobacillus. Graphical representation of real-time qPCR results for the primer set Lact to detect genus *Lactobacillus*, tested using DNA of control bacteria, *Lactobacillus infantis*, and *Bifidobacterium animalis* subspecies *lactis.*



Total Bacteria. Graphical representation of real-time qPCR results comparing primer sets to detect total bacteria.

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APPENDIX C

Measurements of ZDF rats subjected to high-fat diets containing prebiotics and probiotic for three weeks

	CON	FOS	BIF	LAC
Total food intake (*g*)	391.7±8.4	410.9±8.7	394.8±10.0	402.1±14.1
Final blood glucose (*mg/dl*)	270.0±34.6	241.2±44.1	306.0±35.9	320.0±9.5
Body weight gain (*g*)	118.2±4.0	114.8±6.1	113.7±4.6	114.8±3.7
Final body lipid (*%*)	48.2±2.7	45.9±1.9	59.5±3.1	46.1±3.7

Data represent treatment means ± standard error. Letters indicate significant difference between groups as determined from one-way ANOVA and Tukey HSD multiple comparison.

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