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Diet, Prebiotics and Probiotics: Effects on Gut Microbiota in Obesity and Metabolic Disorders

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DIET, PREBIOTICS, AND PROBIOTICS: EFFECTS ON GUT MICROBIOTA IN OBESITY AND METABOLIC DISORDERS

BY

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DISSERTATION ACCEPTANCE PAGE

Thamer Aljutaily

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This dissertation is dedicated to all my valuable treasures in life, particularly to my beloved mother, Miznah Aljutaily; to my lovely wife, Khawlah Aljubaili; to my sweet children, Fahad, Aseel, and Danah; also to my brothers and sisters.

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LIST OF ABBREVIATIONS

BMC:	Bone Mineral Content
BMI:	Body mass index
DXA:	Dual-energy X-ray absorptiometry
FAH:	Food at home
FAFH:	Food away from home
GF:	Germ-free
Gp49b:	Glycoprotein
GI:	Gastrointestinal
GTT:	Glucose tolerance test
GOS:	Galactooligosaccharide
GMT:	Gut microbiome transplantation
HAMSA:	High-amylose maize starch acetylated
HAMSB:	High-amylose maize starch butyrylated
HAMS:	High-amylose resistant starch
HF:	High fat
HDAC:	Histone deacetylase

HbA1C:	Glycated hemoglobin
IL:	Interleukin
IGRP:	Islet-glucose-6-phospatase catalytic subunit-related protein
LF:	Low fat
MHC-I:	Major histocompatibility complex class-I
MyD88:	Myeloid differentiation primary response 88
NOD:	Non-obese diabetic
NF-κB:	Nuclear factor kappa beta
OTU:	Operational taxonomic unit
PCR:	Polymerase chain reaction
PCoA:	Principal coordinates analysis
Ptgs2:	Prostaglandin-Endoperoxide Synthase 2
PP:	Plant polysaccharide
Reg3b:	Regenerating Islet-Derived 3 Beta
STZ:	Streptozotocin
SPF:	Specific pathogen-free
SCFAs:	Short-chain fatty acids

T1D:	Type 1 diabetes
T_{reg}:	Regulatory T cell
WG:	Whole grains
WC:	Waist Circumference

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ABSTRACT

DIET, PREBIOTICS, AND PROBIOTICS: EFFECTS ON GUT MICROBIOTA IN OBESITY AND METABOLIC DISORDERS

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Targeting gut microbiota with diet, prebiotics, and probiotics are emerging as a promising intervention in the comprehensive nutritional approach to reducing obesity and metabolic disorders. Recent human and animal studies suggest that such intervention can promote health benefits by influencing the aspects of metabolism and immunity. However, study of the multi-role association between the diet, the host and the microbiota remains to be clarified. My dissertation attempts to clarify the problem of how gut microbiota (taxonomic composition and predicted functional capacities) affects obesity and metabolic disorders.

In chapter 2, I conducted a placebo-controlled intervention clinical trial to evaluate effects of a synbiotic supplement containing *Bifidobacterium* and *Lactobacillus* strains on the human gut microbiota in relation to changes in body composition and metabolic biomarkers in obesity, followed the weight loss program eating plan (a low-carbohydrate, high-protein dietary pattern with reduced energy intake). The results obtained and bioinformatic analysis support the conclusion that the synbiotic supplement used in this study modulates the human gut microbiota by increasing abundance of beneficial microbial genera and that the supplement may also have beneficial effects on metabolic parameters in obesity.

In chapter 3, I characterized the effect of dairy products (cow, goat, and camel milk and fermented cheese and yogurt originated from cow milk and containing the well-established probiotic *Clostridium butyricum*) on taxonomic composition and relative abundance of the mouse gut microbiota and body weight. The results obtained and their bioinformatics analysis appear to support the conclusion that camel milk and the probiotic cow cheese induce changes the mouse gut microbiota, which are associated with the optimal weight gain in growing mice.

In chapter 4, I evaluate the effect of food at home (FAH) and food away from home (FAFH) diets on human gut microbiota. Substantial work has been done to study whether the FAH diet-induced microbial and immunity changes can protect mice against diabetes. The results obtained and their analysis suggest that the FAH can help to reduce risk of developing diabetes by increasing abundance of potentially beneficial microbial species, T regulatory cells, and decreasing IL-17 producing cells and blood glucose levels.

This dissertation explores a scope of studies on the effects of diet on gut microbiota and health outcomes including obesity and metabolic disorders taking into perspective diets such as food at home, dairy products, high-protein, low-carbohydrate, prebiotic and probiotic. The main outcome of our studies is identification of an effective and novel approach for the prevention and treatment of obesity and metabolic disorders that is based on modulating the human/mouse gut microbiota and increasing abundance of the microbial species that can be considered to be of benefit to their immune system and host.

CHAPTER 1. Introduction and Literature Review

The rising prevalence of obesity and metabolic disorders and its harmful health consequences are of increasing global concern [1, 2]. Recent studies have implicated the gut microbiota (a dynamic and complex population of microbes that live in the gastrointestinal tract) in contributing to this epidemic [3-5]. However, advances in "next generation" DNA sequencing have dramatically increased our capacity to study microbial communities associated with human body habitats (microbiota) [6, 7]. Subsequent studies revealed that gut microbiota influence caloric intake, intestinal absorption, energy balance, and immunological status, therefore strategies aiming at modulating the microbiota to control obesity and metabolic disorders are the focus of considerable attention [8, 9]. Indeed, recent studies have shown that certain prebiotics and probiotics (both diet-based processes that can be used to increase health of the host by enhancing the composition of colonic microbiota) have been successful in promoting weight-loss in diet-induced obesity or diabetes [10, 11].

Importantly, the gut microbiota provides the human host with a number of benefits besides energy extraction, including vitamin synthesis and a reduction of low-grade chronic inflammation associated with obesity and metabolic disorders [12, 13]. Therefore, a healthy and diverse gut microbiota is critical for the well-being of the host [14, 15]. Consequentially, a gut microbiota intervention (either pre- or probiotics or a synbiotic treatment) in individuals suffering a microbial dysbiosis from obesity or severe diets may provide beneficial effects behind weight loss **(Figure 1-1)**.

In these studies, human obese subjects and mouse model were used to test the **hypothesis** that the specific diets, prebiotics and probiotics used in these studies will

modulate the gut microbiota by increasing abundance of the beneficial microbial species, T regulatory cells, and decreasing in inflammation-associated bacteria, IL-17 producing cell populations, and the supplement may also have beneficial effects on body composition and metabolic parameters in obesity. Three specific aims have been designed to test this hypothesis.

AIM 1. To evaluate effects of the synbiotic supplement containing *Bifidobacterium* and *Lactobacillus* strains on the human-gut microbiota in relation to changes in body composition and metabolic biomarkers in obesity.

AIM 2. To characterize the effects of probiotic-enriched pasteurized milk, Greek-style yogurt, and cottage cheese made from milk of different origins (cow, goat, and camel) on the taxonomic composition of the mouse gut microbiota and body weight.

AIM 3. To study the effects of food at home and food away from home diets on human gut microbiota. In addition, to study whether the food at home-diet-induced microbial and immunity changes can protect mice against diabetes.

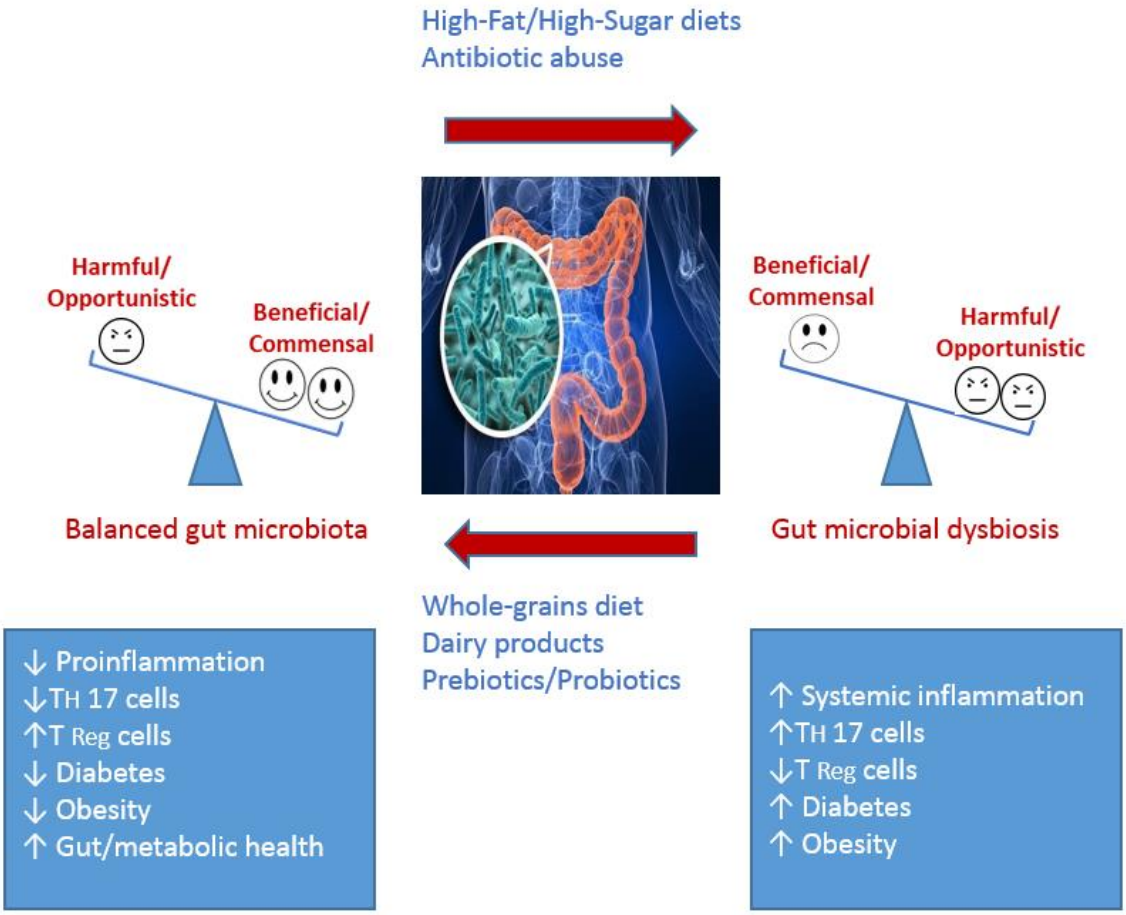

Figure 1-1. Importance of balanced nutrition and gut microbiota. There are shifts in the composition of the microbiota (dysbiosis) whereby either the numbers of Beneficial/Commensal are reduced and/or Harmful/Opportunistic are increased. Several causes (i.e., dietary shifts, sedentary habits, high-fat diet, and antibiotic abuse) can trigger this change and result in non-specific inflammation which can lead to diabetes, obesity, and inflammatory diseases. Healthy diet and lifestyle, whole grains, dairy products and prebiotic/probiotic supplements can reverse this inflammatory status to a healthy anti-inflammatory environment.

Literature Review

The part (1.1.3) included in this literature review have been previously published in the Cellular & Molecular Immunology:

Aljutaily, T., Consuegra-Fernández, M., Aranda, F., Lozano, F., & Huarte, E. (2018). Gut microbiota metabolites for sweetening type I diabetes. *Cellular & molecular immunology*, *15*(2), 92–95. PMID: 28757611. Impact Factor (2018), 8.21; Ranking (2018), 14/158 in Immunology.

1.1.1 Early mice studies

A causal link between gut microbiota and obesity was initially suggested based on studies with germ free (GF) mice. Because gut microbiota has the capacity to ferment otherwise-indigestible dietary compounds, and thus increase energy extraction from foods, GF mice are leaner than conventional mice. However, they rapidly expand their fat mass and increase insulin resistance after colonization with cecal microbiota from conventional mice despite reduce food intake [16, 17]. Subsequent studies demonstrated that colonization of GF mice with cecal microbiota from obese mice resulted in a greater increase in body fat when compared with microbiota transplants from lean mice [18, 19], thus demonstrating that an "obese microbiota" has an increased capacity to harvest energy from the diet, and thus was responsible, and not just the consequence, of metabolic disease and obesity. A more recent study where GF mice were colonized with fecal samples obtained from adult female twin pairs discordant for obesity confirmed the capacity of certain bacterial communities to increase total body and fat mass, as well as obesity-associated metabolic phenotypes. Importantly, the study also demonstrated that the induced metabolic disease was reversible when mice were exposed to a "lean

microbiota" and dependent on the diet [20], thus confirming transmissible, rapid and modifiable effects of diet by microbiota interactions and opening the door to microbiota modification strategies as a therapy against obesity and metabolic disease.

1.1.2 Human gut microbiota

Human beings host trillions of microbes from all domains of life (eukaryota, bacteria, virus and archaea) on multiple surfaces, with the highest density and diversity located on the colon, where bacteria cells are present in concentrations of 10^9-10^{12} per ml [21]. While the diversity of the gut microbiota is mind-blowing (averaging more than 1000 different species with an aggregate of approximately 0.5 million genes per human host), up to 90% of the bacterial species belong to just 2 phyla: *Firmicutes* and *Bacteroidetes* [22, 23]. Originally, a higher proportion of *Bacteroidetes* over *Firmicutes* have been correlated with a leaner status both in mice and humans [24]. Recent studies, however, focus on characterizing the microbiota at the species and strain levels, allowing for finer associations between microbiota and weight. Despite the large inter-individual (and even intra-individual) variation in the gut microbiota, it has also been suggested that individuals can be assigned to one of just 3 different metagenomics profiles (called enterotypes), dominated by either *Bacteroides*, *Prevotella* or *Ruminococcus*, of which *Bacteroides* and *Ruminococcus* are often associated with a low-grade persistent inflammation, obesity and metabolic disease [25, 26].

1.1.3 Gut microbiota metabolites and type I diabetes

Type 1 diabetes (T1D), also referred to as insulin-dependent diabetes mellitus, is a debilitating disease that follows the destruction of pancreatic insulin-producing β cells by autologous T-cells. T1D primarily affects children and has a strong genetic component,

with >50 susceptibility loci identified, including HLA-DQβ chains [27]. However, the striking differences between European regions with similar genetic backgrounds and a sharp rise in T1D incidence in developed countries over the last several decades suggest that environmental factor(s) also play a relevant etiopathogenic role [28]. Given the accumulating evidence linking the gut microbiota to protection against metabolic diseases [29], Mariño *et al.* recently tested the hypothesis that short-chain fatty acids (SCFAs), which are the end products of fermentation of dietary fibers by anaerobic intestinal microbiota, can protect genetically susceptible mice from developing T1D [30]. Interestingly, the authors found that diets enriched in acetate or butyrate (two of the main SCFAs) can protect animals from developing diabetes through different and complementary cellular mechanisms. Such findings significantly enhance our understanding of the role of diet and gut microbiota in the development of autoimmune diseases and indicate that the use of medicinal foods may be a cost-effective treatment against T1D and other autoimmune diseases with a cellular component. Given that current anti-T1D approaches (which focus on prevention or modulation of the adaptive specific immune response against autoantigens) have been generally disappointing [31], such a new and refreshing strategy has attracted a great deal of attention (reviewed in Ref. [28]).

Over the last decade, an ever-growing body of evidence has established that the gut microbiota is one of the most important epigenetic determinants of prevalent metabolic disorders such as type 2 diabetes and metabolic syndrome [32]. Similarly, accumulating experimental observations indicate that T1D incidence in non-obese diabetic (NOD) mice is influenced by the microbial environment, thus indicating that the

gut microbiota is involved in T1D development [33]. This concept has been well illustrated by a recent report demonstrating that the interaction between the gut microbiota and the host immune system was essential for the prevention and treatment of T1D [34]. In their study, Wen *et al.* generated myeloid differentiation primary response 88 (MyD88)-deficient mice in a NOD background (NOD.*Myd88*$^{-/-}$). MyD88 is a master regulator of immune responses and is capable of detecting bacteria and other infectious agents by binding to Toll-like receptors and initiating a pro-inflammatory cascade dependent on nuclear factor kappa beta (NF-κB) activation. NOD.*Myd88*$^{-/-}$ mice kept under specific-pathogen free conditions were completely protected against T1D development, but this protection was dependent on commensal microbiota, as NOD.*Myd88*$^{-/-}$ mice housed in germ-free (GF) conditions developed robust diabetes. Importantly, when these animals were colonized with altered Schaedler's flora, which is a consortium of six bacteria that are normally found in the human gut, protection against T1D was restored. Molecular analyses of cecal microbiota revealed that MyD88 ablation correlates with changes in the microbiota composition, with a significant increase in butyrate-producing *Firmicutes,* as well as *Rikenellaceae* and *Porphoromadaceae*.

SCFA (namely, acetate, butyrate and propionate) are the main metabolites of the bacterial fermentation of dietary fiber and have been associated with anti-inflammatory effects via the up-regulation of regulatory T (T_{reg}) cells and the inhibition of histone deacetylase (HDAC) activity [35]. Given the instrumental role of SCFA in intestinal homeostasis, Mariño *et al.* compared the SCFA concentration in diabetes-prone NOD mice and their diabetes-resistant NOD.*Myd88*$^{-/-}$ counterparts. Although the propionate levels were similar between both mouse lines, the acetate and butyrate levels were much

higher in the NOD.*MyD88*$^{-/-}$ animals, which suggested that T1D protection was mediated by SCFAs. In addition, T1D-prone NOD mice under GF conditions developed a more aggressive form of the disease, thus supporting the role of commensal bacteria-produced metabolites as a defense mechanism against diabetes **(Figure 1-2).**

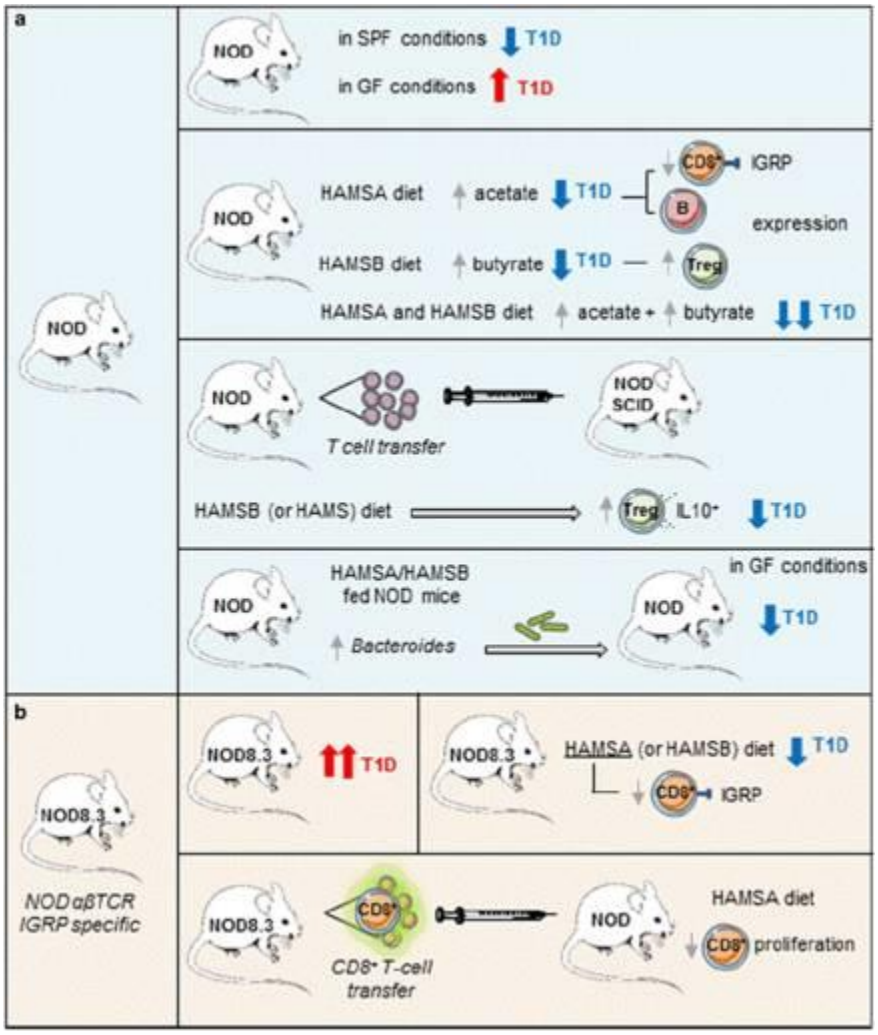

Figure 1-2. Main experimental approach revealing the specific mechanisms of acetate and butyrate protection in T1D development. (**a**) NOD mice undergo exacerbated T1D in germ-free (GF) conditions. Oral administration of acetylated high-amylose maize starch (HAMSA) or butyrylated high-amylose maize starch (HAMSB) increased acetate or

butyrate concentrations, respectively, which protected NOD mice from T1D development via independent mechanisms. Whereas HAMSA administration reduced specific IGRP (islet-specific antigen glucose-6-phospatase catalytic subunit-related protein) reactive $CD8^+$ T-cells and induced a reduction in MHC-I and CD86 expression, HAMSB increased the T_{reg} cell population. Splenic T-cells from NOD mice that were orally treated with HAMSB, when transferred into immunodeficient NOD-SCID mice, reduced T1D severity in receptor NOD-SCID mice. Fecal transplant of gut microbiota from NOD mice fed the HAMSA/HAMSB diet into GF NOD mice transferred diabetes protection. (**b**) NOD mice that express a transgene encoding the αβ TCR derived from a $CD8^+$ T-cell clone specific against IGRP (NOD8.3) undergo acute T1D. The HAMSA diet inhibits the specific proliferation of IGRP reactive $CD8^+$ T-cells NOD8.3 and, ultimately, T1D progression. In an effort to provide both a mechanistic explanation and to increase the potential clinical relevance of their findings, Mariño *et al.* fed NOD mice with special diets designed to release large amounts of specific SCFAs after bacterial fermentation. As expected, mice fed acetylated high-amylose maize starch (HAMSA) showed higher concentrations of acetate, whereas mice fed butyrylated high-amylose maize starch (HAMSB) presented higher concentrations of butyrate. Notably, both diets induced local and systemic increases in the corresponding SCFA levels, but had no effect on body weight or food or energy intake. Confirming their hypothesis that bacterial metabolites can protect genetically prone animals from developing T1D, animals fed either HAMSA or HAMSB presented a significantly reduced incidence of diabetes. Mice fed a combination of these diets demonstrated an even higher protection against diabetes, suggesting different mechanisms of action for acetate and butyrate. This finding was

further confirmed in experiments using NOD8.3 mice, which express a transgene encoding the αβ T-cell antigen receptor derived from a $CD8^+$ T-cell clone specific against islet-specific antigen glucose-6-phospatase catalytic subunit-related protein (IGRP), which is a major target of autoreactive T-cells in pancreatic β-cells [36]. Even in this model of aggressive and rapidly progressing disease, a diet designed to release large levels of acetate showed a protective effect, as evidenced by both a delay in diabetes progression and a diminished percentage of IGRP-specific $CD8^+$ T-cells **(Figure 1-2).**

The authors also found a remarkable reduction in the number of B cells from the spleen and Peyer's patches from NOD mice that were fed the HAMSA diet. Moreover, B-cells from spleen from animals fed the HAMSA diet also expressed lower levels of major histocompatibility complex class-I (MHC-I) and costimulatory CD86 molecules. These results strongly indicate that impaired antigen presentation likely causes a reduction in autoreactive $CD8^+$ T-cells and concomitant protection against diabetes. These results fully agree with the previous report from the same group showing that cross-presentation by antigen presenting B-cells of islet-derived autoantigens drives the expansion and differentiation of self-reactive $CD8^+$ T-cells in the pancreatic lymph node into effector cells, a critical process for the transition from clinically silent insulinitis to overt diabetes [37].

Butyrate has been linked to an increase in the number and activation status of T_{reg} cells [38]. Because T_{reg}s cells are known to play a critical role in controlling T1D [39], the authors next tested the hypothesis that HAMSB-fed animals would have increased numbers of T_{reg} cells and would thus prevent autoreactive T-cells from inducing T1D. In an elegant reverse protection approach, spleen T-cells from NOD mice fed with the

different diets were transferred into severe combined immunodeficiency mice (NOD-SCID). Because SCID mice are depleted of B- and T-cells, the experiment allowed the authors to study the direct effects of the individual diets on the donor T-cells. In this rapidly progressing T1D model, the authors found that the adoptive transfer of spleen T-cells derived from animals fed the HAMSB (but not HAMSA) diet almost completely protected host animals from diabetes development. Surprisingly, spleen T-cells obtained from animals fed with the original high-amylose resistant starch (HAMS) were also protective, albeit to a lesser extent. Further analysis showed that HAMSB-fed animals promoted the conversion of $CD4^+$ T-cells into $Foxp3^+$ IL-10-producing (T_{reg}) cells. Although this result supports a putative role of T_{reg} cells in butyrate-mediated protection against diabetes, a formal demonstration that such T_{reg} cells are indeed responsible for ablating diabetes by inhibiting autoreactive T-cell proliferation was not provided **(Figure 1-2).**

Finally, the authors analyzed changes in the gut microbiota of animals fed with the specialized diets. As expected, NOD mice fed the HAMSA and, to a lesser extent, the HAMSB diets presented a higher percentage of *Bacteroides*—a genus that has been linked with diabetes protection [34]. Importantly, fecal transplant of gut microbiota from NOD mice fed with the acetate-rich HAMSA diet into GF NOD mice was sufficient to elevate levels of acetate and transfer diabetes protection, thus further highlighting the relevance of gut microbiota in T1D pathogenesis **(Figure 1-2)**. However, from a translational point of view, fecal transplants are associated with technical challenges and an almost overwhelming physiological stress and social stigma [40]. Thus, future

experiments should address whether individual bacterial species isolated from colonized GF could also transfer diabetes protection.

Collectively, the data presented by Mariño *et al.* [30] highlight that acetate and butyrate, which are two of the main products of bacterial fermentation, can provide disease protection in a mouse model of autoimmune diabetes. Because each metabolite acts through different molecular mechanisms (acetate reduces the proliferation of autoreactive T-cells by minimizing B cell antigen presentation to T-cells, whereas butyrate increases the number and activity of T_{reg} cells), their additive effects could be beneficial for controlling other immune-based disorders, particularly those of gastrointestinal origin (for example, chronic inflammatory bowel diseases such as Crohn's disease or colitis ulcerosa). Investigations in this regard are warranted and are largely fueled by the increasingly accepted role of the gut microbiota in the development and control of several metabolic diseases, as well as the growing interest in the potential use of prebiotics and probiotics as therapeutic tools to improve gut integrity [41]. The sheer complexity and inter-personal variation of the gut microbiota make any attempt to manipulate it extremely challenging. However, this study opens the door for the use of medicinal food (nutraceuticals) that is rich in bacterial metabolites as a promising and cost- effective treatment against T1D and other autoimmune diseases.

1.1.4 Association between gut microbiota and metabolic health

The gut microbiota of lean and obese individuals was first compared in a seminal study by Ley, *et al.* in 2006 [42] using 16S rRNA sequencing (the 16S rRNA gene is a section of prokaryotic DNA found in all bacteria that codifies for the small subunit of the ribosome and sequencing of its hypervariable regions allows us to identify bacterial

species at various taxa levels). Sequencing of DNA extracted from fecal samples demonstrated that obese individuals present a higher level of *Firmicutes* and lower level of *Bacteroidetes* than lean individuals [42], which validated previous murine studies [43]. Subsequent studies revealed that the gut microbiota composition of obese individuals is less diverse than in lean individuals [44, 45]. However, different results have also been reported [46, 47], and debate continues regarding the significance of the *Firmicutes* and *Bacteroidetes* proportion. Recent meta-analysis study has shown that the microbial changes associated with obesity are not based on phylum differences, but rather are the consequence of numerous small differences within the bacterial community [48]. Therefore, it is important to understand the overall composition of the gut microbiota rather than simply the proportion of *Bacteroidetes* to *Firmicutes*. Importantly, not only different bacteria, but also the metabolites they produce (postbiotics) can play a role in obesity and metabolic syndrome [49]. For example, patients with type-2 diabetes have reduced levels of SCFA-producing bacteria [50, 51], and some SCFAs (e.g., butyrate) facilitate enhanced insulin sensitivity and fatty-acid oxidation in muscle as well as increased satiety [52, 53]. Because obesity has been linked to less diverse gut microbiota composition than in lean individuals, it may be important to increase its diversity, and nutritional approaches to do so (via combined probiotic with prebiotic consumption) can promote weight loss.

According to Brown, *et al*. there is a relationship between the gut microbiota and diseases such as obesity and type 2 diabetes, which are mainly influenced by diet taken [54]. They indicated that since gut microbiota participates in the body's metabolism, energy balances and metabolism of glucose are influenced. Obesity is linked with

dysbiosis (a microbial imbalance) in the intestinal of human beings, therefore there is a high propensity that diet influences dysbiosis and host metabolism [55, 56]. Therefore, gut microbiota has vital roles in host metabolism as well as directing immune system [57, 58]. Other studies have linked gut microbiota with type 2 diabetes since it has been asserted to resist insulin [59]. Therefore, gut microbiota has the propensity of influencing type 2 diabetes in collaboration with or obesity.

1.1.5 Current strategies to modulate the gut microbiota to treat obesity and metabolic disorders

Lifestyle modifications are an important part of obesity prevention and management. Unfortunately, too often they fail to consistently lead to appreciable weight loss [60]. Other alternatives such as pharmacotherapy and surgery can be indicated for short term weight management in severely obese patients, but significant risks and high price prevent them from being the treatment of choice for a majority of patients. Encouraged by its efficacy to treat *Clostridium difficile* infections [61], fecal gut microbiome transplantation (GMT) has been proposed as an alternative for patients that fail to manage weight under other treatments [62]. However, the use for GMT as a treatment for metabolic diseases is only experimental and security concerns as well as psychological stress makes it unappealing [63]. Therefore, it is clear that new approaches are needed to complement existing ones.

Erejuwa, *et al*. indicate that gut microbiota have numerous roles that comprise of digestion, metabolism, and the prevention of pathogens [64]. The authors indicate that there have been a rise in prevalence of metabolic disorders such as obesity, and type 2 diabetes. Therefore, in recent times there has been numerous studies that have linked gut

microbiota with some pathophysiological and the role that gut microbiota plays in metabolic disorders [65, 66]. Since there is a close association of gut microbiota and the metabolic disorders such as obesity, there have been numerous ways that have been suggested to modulate gut microbiota so as to manage the metabolic diseases. Some of the modulation suggested comprise of probiotics and probiotics [64, 67]. For instance, some scientific study on mice found that administering prebiotic supplements in obese mice have the propensity of improving the tolerance of glucose and the content of muscle content [68].

1.1.6 The role of probiotics and prebiotics in health enhancement

Other studies have suggested that probiotics and prebiotics can be used to enhance health of individuals and mitigate chronic diseases such as diabetes type 2 and obesity [49, 69]. Prebiotics and probiotics are diet-based processes that can be used to health of the host by enhancing the composition of colonic microbiota. For instance, prebiotic and probiotic have been found to vary the overall composition of the gut microbiota if the dietary intervention are carried out. Some studies have indicated that prebiotics are dietary substrates that can enhance proliferation or activity of the bacteria that are present in the original colon. Prebiotic in the recent times have been defined as "selectively fermented ingredients that result in specific changes, in the composition and/or activity in the gastrointestinal (GI) microbiota, thus conferring benefit(s) upon host health" [70]. Numerous studies have validated the concept of prebiotic and the role that prebiotics play in minimizing the risk of bowel obstruction and other systematic diseases [71, 72]. Specifically, it was found that prebiotics have a strong potential of protecting human

beings from various chronic diseases or conditions such as type 2 diabetes and obesity [72, 73].

1.1.7 The role of diet in gut microbiota and health outcomes

A wide body of research has been explored to investigate the role of diet on gut microbiota and the resulting health outcomes especially type 2 diabetes and obesity among with the health complications linked to these diseases. Conlon & Bird (2014) investigated the impact of diet and lifestyle on gut microbiota as well as the human health in a study that recognizes that diet is an important factor in regulating the composition and metabolic activity of gut microbiota [69]. Gut microbes are known to produce numerous number of bioactive compounds that can impact the health of an individual, therefore dietary means such as the consumption of fibers can be very vital in maintenance of a healthy gut microbiota population [69, 74]. Gut microbial have the capability of producing numerous products, and some of the products produced have adverse effect on the health of human beings [75]. The impact of diet in the gut microbiota and health has been vastly studied, for instance diets rich in fiber have been found to promote and maintain bowel health due to the fact that they increase digested mass [69]. Additionally, dietary fat have been found to influence the structure as well as the metabolic activity of gut microbiota, and this phenomenon has been associated with obesity [69, 76]. Studies indicate that high fat diets triggers circulation levels of bacteria that are associated with lipopolysaccharide (LPS), and this is can be attributed to an enhancement of intestinal permeability [77].

Graf, *et al*. (2015) indicated how important the human gut microbiota is, and the manner in which it is influenced by various factors such as diet [78]. The authors

indicated that nutrients that are indigestible for human enzymes such as fiber provides substrates for the intestinal microbial metabolism. The fact that bacteria are responsible for triggering the fermentation of different substrates, complex diet can either promote or inhibit growth for some phylotypes [79]. For instance whole grain products are mainly characterized with high content of fiber, and the fact that the human body doesn't have any enzymes that can digest their structures they reach the colon where they are metabolized by the microbiota, which in turn inhibit the growth of different bacterial groups [80, 81]. The composition of the human gut microbiota can be argued to be stable and any short term dietary intervention does not seem to change the composition of the microbiota. Nonetheless, the microbial gene expression and consequently the functional profiles seems to adopt to changes in diet more easily [80, 82].

Heinritz, *et al*. (2016) conducted a research to ascertain the impact of high-fat or high fiber on intestinal metabolic markers where they used pigs in their study [83]. The main reason why the pigs were used is because they have the same dietary changes similar with those of humans. The study ascertained that there was a hypotrophic effect on the high-fat (HF) diet compared to low-fat (LF) diet. Diets rich in fiber have also been reported to be trophic to their ability to ferment easily, thereby enhancing production of SCFA that stimulates epithelial cell proliferation [83, 84]. Metabolic activity of gut microbiota is mainly influenced by the production of different microbial metabolites, for instance consumption of LF diet have the propensity of increasing the concentration of butyrate and acetate in cecum and colon [83, 85]. Therefore, the role of diet in regard to the composition and the metabolic activity of gut microbiota is very important since it has the propensity of influencing health [69]. Diet is a vital aspect that shapes gut microbiota,

and this means that diet and nutritional components should be taken into consideration since they can trigger diseases such as obesity [83]. For instance, some studies have indicated that harmful and pathogenic gut microbes are linked to diseases such as obesity, and this is because some diet causes imbalances of gut microbiota [56, 79]. Thus, different nutrients have different components that impacts gut microbiota, which in turn influences aspects such as the metabolic activity.

According to previous research on the impact of diet on health-type 2 diabetes and obesity, it is worth to note that the adverse metabolic effects of 'western' style of diet characterized by high sugar and salt levels as well as highly processed foods go beyond the macronutrients [86]. For instance, artificial sweeteners and emulsifiers have been linked to the development of metabolic syndrome aspects through the modulation of the microbiota mice [87, 88]. According to the findings from a study conducted by McGill, *et al*. there was evidence of insulin resistance when hosts fed on high doses of artificial sweeteners after only 7 days of intake of the sweetener [89]. These findings provide insights into the conclusion that there is scientific evidence to suggest that artificial food additives, especially those with high sugar level contribute immensely to development of metabolic disease by disturbing the microbiota. This gives rise to the development of diseases such as type 2 diabetes and obesity. Worth noting is the insights provided by the analysis of the differences in susceptibility of the western countries and those in developing countries especially in Africa. For instance, western dietary trends are historically characterized by low levels of dietary fiber based on plants, which is an essential fuel for microbiota [90, 91]. As such, the inclusion of abundant level of nutrients that negatively impact the microbiota in the absence of dietary fiber creates a

situation that makes it easy to understand metabolic diseases as such dietary consumption is the leading causes of diabetes and obesity.

1.1.8 The role of gut microbiota in the regulation of fat storage and homeostasis

Research shows evidence of the influence of diet in the gut microbiota and health especially diabetes and obesity. These two health complications are evidenced by the role of the gut microbiota in the regulation of fat storage and homeostasis [12, 92]. According to a study conducted by Chassaing, et al., (2015), it is reported that gut barrier dysfunction and microbiota alteration are resultant are induced by two dietary emulsifiers; polysorbate-80 and carboxymethylcellulose in mice [93]. As a result, the mice experienced major metabolic disorders, low-grade inflammation, weight gain and rise in blood sugar level. While it is not conclusive that intake of 'western' processed foods in uniquely responsible for the increase in cases of inflammatory diseases and obesity, there is no doubt that consumption of several of such compounds could play a major role in inducing worse metabolic health and low-grade inflammation in organisms that may be susceptible to metabolic disease. A large body of literature relate to the comprehension of the role of gut microbiota in influencing metabolism, absorption and storage of calories after digestion [94, 95]. Research suggests that gut microbes alter the manner in which the human body reacts to elements of diet and nutrition to influence metabolism. However, there is lack of conclusive evidence on the mechanisms that underlie this process as they are highly complex to reconcile. Nevertheless, emerging trends in the field suggests that obesity is linked to reduced diversity of the gut microbiota [96, 97].

1.1.9 Gut microbiota and sources of dietary fats

Most of the experiment that evaluate the role of diet in the gut microbiota and type 2 diabetes, obesity use western type of diet, which is full of processed foods and devoid of fibers [98]. Such foods are filled with calories from the saturation of sucrose and fat, and they illustrate that gut microbiota significantly regulates the occurrence of obesity through additional pathways [99, 100]. For instance, research shows that germ-free mice that have been fed with a diet that is low in sucrose and high in fiber show partial protection from obesity, primarily microbiota-dependent obesity [101]. This type of protection is withdrawn upon the omission of sucrose from the diet. For purposes of this analysis, it is very important that the source of the dietary fats be understood. This is because the two types of dietary fats; saturated and unsaturated deliver diverse influence on the gut microbiota [102, 103]. Moreover, the modified microbiota caused by intake of unsaturated fats plays a role in protecting human beings from weight gain resulting from such feeding [76, 104]. Subsequently, weight gain becomes inevitable with consumption of unsaturated fats than when saturated fats are consumed. The review of previous findings suggest that fats and simple carbohydrates could result in unexpected impacts on the metabolism of users of such diet through microbiota.

1.1.10 Link between gut microbiota, insulin resistance, obesity, and inflammatory reactions

Insulin resistance and obesity have been linked to the rise in the rate of infiltration of inflammation and macrophages in the adipose tissue [105, 106]. As gut microbiota is reported to be a significant contributor of increasing the prevalence of the obese phenotype, mostly in mice as it has been confirmed by several researchers, it is also

deemed to contribute to a high level of adipose inflammation [107, 108]. This type of model where adipose inflammation is independent of diet but dependent on microbiota can be evidenced by the finding resulting from a study conducted using C57Bl6 common laboratory mice. The study reported that the mice exhibited minimized adiposity as well as lower levels of endotoxins in the blood when fed with diets that have been inducted with adiposity. Moreover, the mice demonstrate enhanced level of glucose metabolism and a reduced level of macrophage infiltration in the white adipose tissue. The occurrence of obesity in mice is also reported to be linked to the increase in the amount of mast cells, T cells as well as the reduction in the amount of regulatory T cells [38, 109]. When using the mouse models, there is an indication of generation of short-chain acids and fermentation of fiber, which demonstrates the promotion of anti-inflammatory reactions both through regulatory T cells systematically and within the gut [35, 110]. While interleukin (IL)-17 (an inflammatory cytokine) is involved in the pathogenesis of diabetes and obesity [111, 112]. There is no doubt that the dietary fiber and the short-chain acids produced in the model mice result in a positive metabolic effect through non-immunological strategies [113]. The mouse used for the study are those that had been obesity-induced. However, it is not clear whether the same outcome that would result from the immune system could lead to metabolic alterations. While the processes that underlie these procedures remain unclear, research suggests that when high fat diets are supplements with fermented fiber, the mice are protected from suffering from obesity and its associated diseases and disorders.

It is widely acknowledged that diet, especially fiber and processed foods have a great influence on gut microbiota and consequently on type 2 diabetes and obesity. As

obesity is significantly correlated to the development of low grade chronic inflammation, diabetes and obesity are common resultant health complications due to the contribution of the inflammation on the risk of insulin resistance and body weight. Moreover, individuals risk suffering from other health problem related to obesity including cardiovascular diseases [105]. Obesity is also attributed to modified gut microbiota. This is illustrated by the metabolism of indigestible polysaccharides in a process that produces monosaccharides and short chain acids that allow for such products to be absorbed and stored in the body as fat. The general body of research tying diet to gut microbiota and health outcomes demonstrates that diet low in fiber interacts to effect alternation of the inflammation in the intestines, a move that is followed by other health complications such as insulin resistance, adiposity, and weight gain, hence predisposing individuals to type 2 diabetes and obesity.

1.1.11 Dairy products for probiotic benefits

Manipulation of the gut microbiota through dietary intervention and probiotic supplements is a promising strategy for the prevention and treatment of metabolic diseases. As a consequence, worldwide sales of probiotics have more than doubled in the last decade alone [114]. On the other hand, probiotic bacteria have often been assessed as dietary supplements or in dairy products, such as yogurt or kefir [115, 116]. Because of its nutritional values and longer shelf life, fermented cheese might be an ideal vector for the delivery of probiotics. Cheese is a good matrix for transferring probiotic bacteria into the gastrointestinal tract, due to the buffering capacity of the milk proteins which will protect the cells during transit; this is mainly due to their higher fat content and denser structure that may protect the bacteria against the acidic environment of the

gastrointestinal tract [117-119]. While several studies have shown an inverse association between dairy products and metabolic syndrome [120-122], however, it is not clear whether the same outcome that would result from the different sources of (cow, goat and camel) and formats (milk, yogurt, probiotic yogurt, cheese and probiotic cheese) could lead to gut microbiota alterations. While the processes that underlie these procedures remain unclear, research suggests that when full-fat dairy are supplements with specified probiotic, the mice are protected from suffering from obesity and its associated diseases and disorders.

1.1.12 Links between diet, antibiotics treatment, gut microbiota, and immunological status

Linking diet to the structure and dynamic operation of microbial communities and, more importantly, to the immunological status of the host has proven elusive because of numerous uncontrolled genetic and environmental variables and the intrinsically complicated nature of clinical trials. In the past, germ-free mice (animals born and raise without any exposure to micro-organisms) have provide an excellent system for controlling host genotype, diet and environmental conditions. In addition, they can be colonized at specific life stages with different microbial communities and thus perform comparative metagenomic studies of donor communities [123, 124]. However, raising and maintaining a germ-free mice colony is challenging. Hence, a broad range antibiotic treatment of mice is considered a viable alternative and has been successfully used in the past. Antibiotics have been administered by either oral or intraperitoneal route [125-127]. However, several studies have shown a negative repercussions between antibiotic treatment and composition of gut microbiota, including dysbiosis as well as

metabolic functions and immune responses [128, 129]. While the processes that underlie these procedures remain unclear, research suggests that when diet are supplements with specified probiotic and prebiotic, the mice are protected from suffering from this repercussions as well as attenuating antibiotic-induced disturbances in the gut microbiota composition.

1.1.13 Summary

This review explores a wide scope of literature on the effects of diet on gut microbiota and health outcomes including obesity and metabolic disorders taking into perspective diets such as high fat, whole grains, dairy products, prebiotic and probiotic. High fiber diet as well as diet rich in prebiotic and probiotic is deemed to be effective in mitigating the occurrence of diabetes and obesity as they enhance metabolism and digestion, minimizing the risk of bowel obstruction. Recent human and animal studies suggest that such intervention can promote health benefits by influencing the aspects of metabolism and immunity. However, study of the multi-role association between the diet, host and the microbiota remains to be clarified. My dissertation attempts to clarify the problem of how gut microbiota (taxonomic composition and predicted functional capacities) affects obesity and metabolic disorders, and provided important information on correcting disruption of the gut microbiota resulting from obesity or imbalanced diets using a combine probiotic and prebiotic (synbiotics), and dairy products which can be effectively employed for the delivery of probiotics to the gut as well as for the support of growth and survival of probiotic bacteria.

CHAPTER 2. Effects of Synbiotic Supplement on Human Gut Microbiota, Body Composition and Weight Loss in Obesity

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Abstract: Targeting gut microbiota with synbiotics (probiotic supplements containing prebiotic components) is emerging as a promising intervention in the comprehensive nutritional approach to reducing obesity. Weight loss resulting from low-carbohydrate, high-protein diets can be significant, but has also been linked to potentially negative health effects due to increased bacterial fermentation of undigested protein within the colon and subsequent changes in gut microbiota composition. Correcting obesity-induced disruption of gut microbiota with synbiotics can be more effective than supplementation with probiotics alone because prebiotic components of synbiotics support growth and survival of probiotic bacteria. The purpose of this placebo-controlled intervention clinical trial was to evaluate effects of a synbiotic supplement on composition, richness and diversity of gut microbiota and associations of microbial species with body composition parameters and biomarkers of obesity in human subjects participating in a weight loss program. The probiotic component of the synbiotic used in the study contained *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *B. longum*, and *B.*

bifidum and the prebiotic component was a galactooligosaccharide mixture. The results obtained indicated that there were no statistically significant differences in body composition (body mass, BMI, body fat mass, body fat percentage, body lean mass, and bone mineral content) between the placebo and synbiotic groups at the end of the clinical trial (3-month intervention, 20 human subjects participating in weight loss intervention based on a low-carbohydrate, high-protein, reduced energy diet). Synbiotic supplementation increased abundance of gut bacteria associated with positive health effects, especially *Bifidobacterium* and *Lactobacillus*, and it also appeared to increase the gut microbiota richness. A decreasing trend in the gut microbiota diversity in the placebo and synbiotic groups was observed at the end of trial, which may imply the effect of the high-protein, low-carbohydrate diet used in the weight loss program. Regression analysis performed to correlate abundance of species containing in the synbiotic supplement with body composition parameters and biomarkers of obesity found association between a decrease over time in blood glucose and an increase in *Lactobacillus* abundance, particularly in the synbiotic group. However, the decrease over time in body mass, BMI, waist circumstance, and body fat mass was associated with a decrease in *Bifidobacterium* abundance. The results obtained support the conclusion that synbiotic supplement used in this clinical trial modulates human gut microbiota by increasing abundance of potentially beneficial microbial species and that the supplement may have positive effects on metabolic parameters in obesity.

Keywords: synbiotic; prebiotic; probiotic; gut microbiota; obesity; weight loss; body composition; obesity biomarkers

2.1. Introduction

The gut microbiota appears to play a role in the pathogenesis of obesity and obesity-associated diseases [130, 131]. This community can contribute to the development of obesity primarily by influencing dietary energy intake and intestinal absorption of nutrients [132, 133], but it can also provide the human host with benefits besides energy extraction, including a reduction of low grade chronic inflammation associated with obesity and metabolic syndrome [134, 135]. Therefore, gut microbiota may be considered as a promising target in the comprehensive dietary approach to the prevention and treatment of obesity, including weight loss and weight maintenance [136, 137].

It is important to note that high-protein and low-carbohydrate diets, which are often successfully used for weight loss, have been associated with a decrease in bacteria considered beneficial to health [47, 138, 139]. Those diets have also been found to induce protein fermentation by gut microbiota with formation of metabolic byproducts [140-142], which can trigger inflammation in the colon [143]. Furthermore, high levels of protein fermentation by gut bacteria have been associated with increased genotoxicity [144]. Thus, a healthy, diverse, and less toxic gut microbiota is critical for wellbeing of the host [145, 146].

Dietary intervention with probiotics, prebiotics or synbiotics (which combine probiotic and prebiotic components) aimed at correcting disruption of the gut microbiota resulting from obesity or imbalanced diets may provide health benefits by facilitating weight loss and maintenance [147, 148]. Recent human and animal studies suggest that

probiotics can promote weight loss in obesity [149, 150], but studies on the role of synbiotics in obesity are very limited [151, 152] and further studies are warranted [151].

The objective of this placebo-controlled intervention clinical trial was to evaluate effects of a synbiotic supplement containing *Bifidobacterium* and *Lactobacillus* strains on the human gut microbiota in relation to changes in body composition and metabolic biomarkers in obesity. The results obtained and bioinformatic analysis support the conclusion that the synbiotic supplement used in this study modulates the human gut microbiota by increasing abundance of beneficial microbial genera and that the supplement may also have beneficial effects on metabolic parameters in obesity.

2.2. Materials and Methods

2.2.1. Study Participants, Clinical Trial Design and Prebiotic Supplement

Participants of the study were enrolled in the weight loss program (Profile by Sanford Health, Sioux Falls, SD). Twenty new weight loss participants, male and female, were recruited for the study and randomly assigned to the placebo (control) or synbiotic (treatment) group. Those enrolled were initially overweight/obese and had a mean BMI of 33.5 kg/m^2. Placebo group (n=10) followed the weight loss program eating plan (a low-carbohydrate, high-protein dietary pattern with reduced energy (4,000-5,000 kJ/d) intake). The synbiotic group (n=10) was on the same diet plan, but additionally received a synbiotic (probiotic plus prebiotic) supplement daily for 3 months. The control group received the placebo supplement similar in appearance and of the same energy content as the synbiotic supplement. Human subjects with conditions that may impact gut microbiota (gastrointestinal, autoimmune, and metabolic diseases and medications,

particularly antibiotics) were not included in the trial. All subjects gave their informed consent for participating in the study. The study was approved by the SDSU Institutional Research Board (Approval number: IRB-1604005). The clinical trial has been registered in the ClinicalTrials.gov (NCT number: 03123510).

The probiotic component of the synbiotic used in the study contained a blend (one capsule contained 69 mg or $15x10^9$ CFU) of proprietary strains of *Lactobacillus acidophilus* DDS-1, *Bifidobacterium lactis* UABla-12, *B. longum* UABl-14, and *B. bifidum* UABb-10. The probiotic supplement was produced by UAS Labs (Wausau, WI). The prebiotic component was a *trans*-galactooligosaccharide (GOS) mixture at a dose of 5.5 g/d (2.75 g GOS and the remainder simple sugars) produced by Clasado BioSciences Ltd. (St. Helier, Jersey, UK) [153].

2.2.2. Body Composition and Metabolic Parameters

Body mass index (BMI) was calculated using body weight and height measured with bare feet and in minimal clothing using a stadiometer and an electronic scale. Body composition parameters (body fat mass and percentage, body lean mass, and bone mineral content) were acquired before and after 3 months of synbiotic intervention by dual-energy X-ray absorptiometry (DXA) using a whole-body scanner (Hologic APEX, Bedford, MA)[154]. Whole-body images were obtained and analyzed by a technologist certified as a Certified Bone Densitometry Technologist by the International Society of Clinical Densitometry. Phantom scans were performed before participant testing as an independent assessment of system calibration, and quality control data were plotted and reviewed periodically. The A1CNow$^+$ test (MDSS GmbH, Germany/Polymer

Technology Systems, Inc., USA) was used for quantitative measurement of the percent of glycated hemoglobin (%A1C) in the capillary blood (fingerstick) samples.

2.2.3. Microbial DNA Extraction and the 16S rRNA Gene Sequencing

Fecal samples from the participants were obtained before and after the synbiotic or placebo intervention with no more than 24 h prior to the study visit. Samples were collected using the OMNIgene-GUT stool/feces collection kit (OMR-200, DNA Genotek, Ottawa, Canada). Forty fecal specimens from unique participants were sent to DNA Genotek for the microbiome analysis. DNA was extracted and quantified and library preparation was performed with Illumina's NexteraXT protocol. Aliquots of each sample were extracted using PowerMag microbial DNA isolation kit (MO BIO Laboratories, Carslbad, CA) optimized for the KingFisher Flex automated extraction platform (ThermoFisher, Pittsburgh, PA). A bead-beating step with glass beads was used to maximize recovery of DNA from low-abundance and difficult to lyse microorganisms. The concentration of extracted DNA was measured using Qubit Fluorometer (Invitrogen, Carslbad, CA), and the sample purity was confirmed spectrophotometrically by measuring the A260/A280 ratio.

For DNA sequencing, Illumina sequencing adapters and dual-index barcodes (Nextera XT indices) were added to the amplicon target via polymerase chain reaction (PCR) amplification. 16S sequencing (2x300 bp PE V3-V4) was performed on Illumina's MiSeq platform. Amplicon sequencing was performed to a target depth of 30,000 reads per sample. Paired-end reads from each sample were merged, screened for length and filtered for quality using DNA Genotek's proprietary 16S pre-processing workflow. Read merging and quality filtering was performed on the raw sequencing reads to eliminate

any sequencing artifacts and low quality reads. Complete quality metrics including library quantification and sequencing run quality control are presented in *Supplemental Materials* (**Figure 2-S1 and Table 2-S1**).

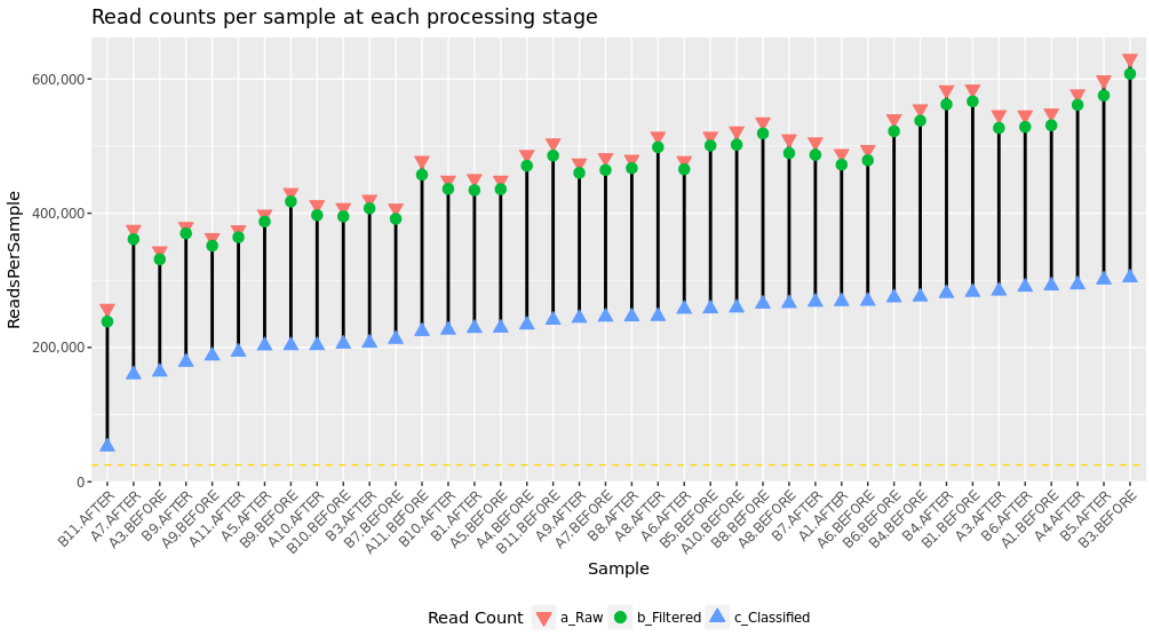

Figure 2-S1. Sequence counts per sample for raw, filtered and classified sequences ordered by increasing classified sequence counts. Dashed gold line indicates the 25,000 sequence threshold used for rarefaction. *A* and *B* before sample numbers indicate the synbiotic and placebo groups, respectively.

Metric	**Minimum Requirement**	**Results**
% ≥ Q30	≥ 75%	82.38 %
Cluster Density	≥ 800 k/mm2	1,111 ± 19 k/mm2
Clusters Passing Filter (%)	≥ 85%	86.49 ± 1.34 %
Sequencing Yield	≥ 13.2 Gbp	14.04 Gbp
PhiX Alignment (%)	12% - 18%	11.63 %

Table 2-S1. Sequence quality metrics per sequencing run. % ≥ Q30: The proportion of base calls that have a confidence score of 30 or more. This is a commonly cited metric that can be used to evaluate the overall quality of a sequencing run. Cluster Density: How efficiently the sequencer is able to bind sequences of DNA to the flow cell. A higher density represents a more efficient sequencing run. Clusters Passing Filter: The proportion of clusters that meet the sequencer's minimum quality threshold for sequence quality. Only clusters that pass filter are included in the sequencer's FASTQ output. Sequencing Yield: Refers to how many nucleotide base pairs were called by the sequencer. 1 Gbp (giga base pair) means the sequencer generated 1 billion base pairs of output. PhiX Alignment: PhiX is a sequencing library that is used as a positive control on each sequencing run. The sequencer aligns reads to the PhiX library to calculate sequence-based quality control metrics. We require that the percentage of reads that align to the PhiX library is within 20% of the spike-in amount of PhiX.

2.2.4. Taxonomic Classification and Bioinformatics Analysis

A curated reference taxonomic database was used to assign a taxonomic classification to the sequencing reads. High quality sequences were aligned to the curated reference database at 97% similarity using the NINJA-OPS algorithm, version 1.5.1 [155]. At 97% sequence identity, each operational taxonomic unit (OTU) represents a genetically unique group of biological organisms. These OTUs were then assigned a curated taxonomic label based on the SILVA taxonomic database, version 123 [156]. The relative abundance of all taxa at the phylum and genus levels were plotted to visualize

sample-specific classifications. All samples were rarefied to an even depth of 25,000 classified sequences per sample or more to eliminate effects of variance in sequencing depth. Samples with more than 25,000 classified sequences per sample were included in the rarefied OTU table and downstream analyses, thus allowing to rarefy the samples to 52,150 read pairs/sample, as this was the read count of the sample with the fewest reads (see **Table 2-S1**).

Alpha diversity metrics (observed OTUs, Shannon index, and Chao1 diversity) were calculated on the rarefied OTU table using the alpha_rarefaction.py workflow in QIIME 1.9.1 [157]. Beta diversity metrics (weighted and unweighted UniFrac distances) were calculated on the rarefied OTU table with the beta_diversity.py workflow in QIIME 1.9.1 and Bray-Curtis dissimilarity index was calculated on the species level summarization of the rarefied OTU table. Differences between groups were estimated using Permutational Multivariate Analysis of Variance (PerMANOVA; adonis function in the vegan R package). Principal Coordinates Analysis (PCoA) was applied to each beta diversity distance matrix using the dudi.pco function from the R package made4 (version 1.48.0). The first two major axes were plotted (R package ggplot2 version 2.2.1), and the percentage of variance explained by each axis was indicated.

2.2.5. Statistical analysis

A one-way ANOVA with independent samples *t*-test was used for group comparison of the body composition and metabolic parameters (SPSS Statistics, v. 25). The results were expressed as mean ± SD, and mean differences were considered significant at $p < 0.05$. Significant differences in alpha diversity between groups were determined using estimated marginal means analysis applied to linear mixed model,

which was built with alpha diversity as the response variable, the treatment groups and time points as the predictor variables, and subject number as a random variable. Significant differences in beta diversity between groups were determined using PerMANOVA with beta diversity as the response variable and the treatment groups and time points as predictor variables. Statistical analyses of diversity metrics were performed using R version 3.3.2 (R Core Team, 2015).

Associations between relative abundance of gut bacteria and the body composition and metabolic parameters were calculated using Pearson's linear correlation coefficient. Regression analysis to correlate microbial abundance of species/genera present in the synbiotic supplement (*Bifidobacterium* and *Lactobacillus*) with body composition parameters and biomarkers of obesity was performed by applying ANOVA to a mixed linear model build with the percent abundance of microbe of interest as the response variable and the interaction between the specific parameter (gender, age, body mass, weight circumstance, BMI, body fat mass, body fat percentage, lean mass, bone mineral content, or HbA1C), treatment groups (placebo or synbiotic) and time points (beginning or end of trial) as predictor variables, with subject number as random variable. The Bonferroni correction method was used for multiple testing. Software versions used for the data analyses are provided in supplemental **Table 2-S2**.

Software	**Version**
Trimmomatic	0.36
NINJA-OPS	1.5.1
SILVA taxonomic database	123
QIIME	1.9.1
SPSS Statistics	25
R	3.3.2
R packages	
emmeans	1.2
lme4	1.1-17
Matrix	1.2-14
scales	0.5.0
ggplot2	2.2.1
reshape2	1.4.3
made4	1.48.0
scatterplot3d	0.3-41
gplots	3.0.1
RColorBrewer	1.1-2
ade4	1.7-11
vegan	2.5-1
lattice	0.20-35
permute	0.9-4

Table 2-S2. Software versions for data analysis.

2.3. Results

In this placebo-controlled intervention clinical trial, effects of the synbiotic supplement on richness and diversity of gut microbiota and associations of microbial species with measurements of body composition and biomarkers of obesity were evaluated in human subjects participating in a weight loss program. Twenty participants were recruited in the study (10 in the placebo (control) group and 10 in the synbiotic (treatment) group). The average BMI of the study participants was 33.5 kg/m^2 and the average age was 47.4 years. The majority of participants were female (80% in the placebo group and 70% in the synbiotic group).

Participants were enrolled in the weight loss program at the beginning of the study and followed a low-carbohydrate, high-protein, reduced-energy intake eating plan. The probiotic component of the synbiotic used in the study contained *Bifidobacterium* spp. and *Lactobacillus acidophilus* strains, and the prebiotic component stimulating growth of these bacteria was a *trans*-galactooligosaccharide mixture. Blood and fecal samples were collected and body composition and metabolic parameters measured at the beginning and end of the three-month intervention trial. Seven human subjects in the placebo group and eight human subjects in the synbiotic group had body composition parameters measured using DXA. No participants dropped out of the study during the intervention period.

2.3.1. Body Composition and Metabolic Parameters

The results obtained indicate that there were no statistically significant differences in the body composition parameters (body mass, waist circumstance, BMI, body fat mass, body fat percentage, body lean mass, bone mineral content (as measured by DXA) and

obesity-related biomarkers (blood glucose, as measured by HbA1C levels) between the placebo and synbiotic groups at the end of the clinical trial (three-month synbiotic intervention) (**Table 2-1**). Body mass, waist circumference, BMI, fat mass, fat percentage, and glucose level significantly decreased or demonstrated a decreasing trend in the placebo and synbiotic groups at the end of the trial (participants in both the placebo and synbiotic groups were enrolled in the weight loss program). The decrease in HbA1C percentage at the end of trial was statistically significant in the synbiotic group, but not in the placebo group. Individual body composition parameters, including the DXA scan measurements, are presented in appendix Table 2-S3.

Characteristics/Parameters	**Placebo**	**Synbiotic**	***p***
Sex (%)			
Male	20.0	30.0	
Female	80.0	70.0	
Age (years)	47.0 ± 15.4	47.8 ± 8.99	0.88
Height (cm)	171.8 ± 12.9	163.4 ± 9.63	0.30
Body mass (kg)			
Baseline	97.6 ± 23.1	90.6 ± 11.9	0.40
End of trial	90.0 ± 21.9	83.4 ± 11.4	0.41
Body mass change (%)	7.78 ± 5.30*	7.94 ± 3.88*	0.86
BMI (kg/m^2)			
Baseline	32.77 ± 4.51	34.20 ± 5.60	0.53
End of trial	30.14 ± 4.04	31.48 ± 5.23	0.53
BMI change (%)	8.02 ± 1.65*	7.95 ± 1.52*	0.82
WC (cm)			
Baseline	106.9 ± 12.47	109.6 ± 8.07	0.57
End of trial	101.1 ± 12.89	102.6 ± 8.48	0.76
WC change (%)	5.42 ± 5.78*	6.38 ± 4.16*	0.29
Body Fat Mass (kg)			
Baseline	40.66 ± 6.92	36.97 ± 11.35	0.47
End of trial	37.44 ± 6.99	34.06 ± 11.58	0.51
Fat mass change (%)	7.91 ± 2.73*	7.87 ± 3.94*	0.37
Body Fat (%)			
Baseline	40.97 ± 5.02	40.51 ± 8.96	0.90

End of trial	39.51 ± 4.53	39.13 ± 9.47	0.92
Body fat change (%)	3.56 ± 1.49*	3.40 ± 2.97	0.20
Body Lean Mass (kg)			
Baseline	57.39 ± 17.76	51.13 ± 8.87	0.39
End of trial	55.61 ± 16.15	49.47 ± 8.64	0.36
Lean mass change (%)	3.10 ± 2.10*	3.24 ± 1.14*	0.25
BMC (kg)			
Baseline	2.66 ± 0.64	2.38 ± 0.48	0.34
End of trial	2.68 ± 0.67	2.38 ± 0.48	0.32
BMC change (%)	0.75 ± 0.05	0.16 ± 0.01	0.10
Body Lean Mass + BMC(kg)			
Baseline	60.05 ± 18.38	53.52 ± 9.35	0.39
End of trial	58.30 ± 16.78	51.86 ± 9.11	0.36
Lean mass +BMC change (%)	2.91 ± 2.08*	3.10 ± 1.13*	0.26
HbA1C (%)			
Baseline	5.36 ± 1.07	5.39 ± 0.28	0.93
End of trial	5.06 ± 0.37	5.06 ± 0.43	1.00
HbA1c change (%)	5.59 ± 0.89	6.12 ± 0.47*	0.24

Table 2-1. Characteristics of study participants at the beginning and end of the intervention clinical trial. BMI, Body Mass Index; WC, Waist Circumference; BMC, Bone Mineral Content; HbA1C, glycated hemoglobin. The duration of the trial was 3 months. The study enrollment period was 6 months, and subjects were assigned to the groups in a chronological order. n = 10 for the placebo group and n = 10 for the synbiotic group (7 human subjects in the placebo group and 8 human subjects in the synbiotic group completed DXA scans). The results are expressed as mean ± SD. A one-way ANOVA with independent *t*-test was used for the group comparison (SPSS Statistics, v. 25). (*), $p < 0.05$, as compared between the beginning (baseline) and end of trail for the same group (placebo or synbiotic); *p* value, as compared between the placebo and synbiotic groups.

The findings obtained demonstrate that the low-carbohydrate, high-protein, decreased-energy diet is effective for weight loss and normalizing obesity-related metabolic parameters (blood glucose), but they do not support the conclusion that the synbiotic supplement used in the study has significant impact on body mass and body composition of human subjects participating in this weight loss program.

2.3.2. Gut Microbiota

To characterize effects of the synbiotic supplement on gut microbiota of the study participants, fecal samples were obtained before and after the synbiotic intervention, gene sequence analysis was performed, and individual variations as well as group differences of gut microbiota were compared. All samples underwent taxonomic classification and were included in the complete OTU table (supplemental **Table 2-S4**), however, those with fewer than 25,000 classified sequences per sample were excluded from further analysis. Remaining samples were rarefied to a depth of 52,150 sequence reads per sample. Raw read counts per sample, quality of filtered read counts per sample, and sequence quality metrics per sequencing run are provided in *Supplemental Materials* (see **Figure 2-S1** and **Table 2-S1**). The relative abundance of all taxa at the phylum, genus, and species levels were plotted to visualize broad taxonomic differences by treatment groups and time points with a percentage of each number in all sequencing reads **(Figure 2-1** and **Figure 2-S2**). In addition, the relative abundance of phyla, genera, and species per sample were plotted (supplemental **Figure 2-S3**).

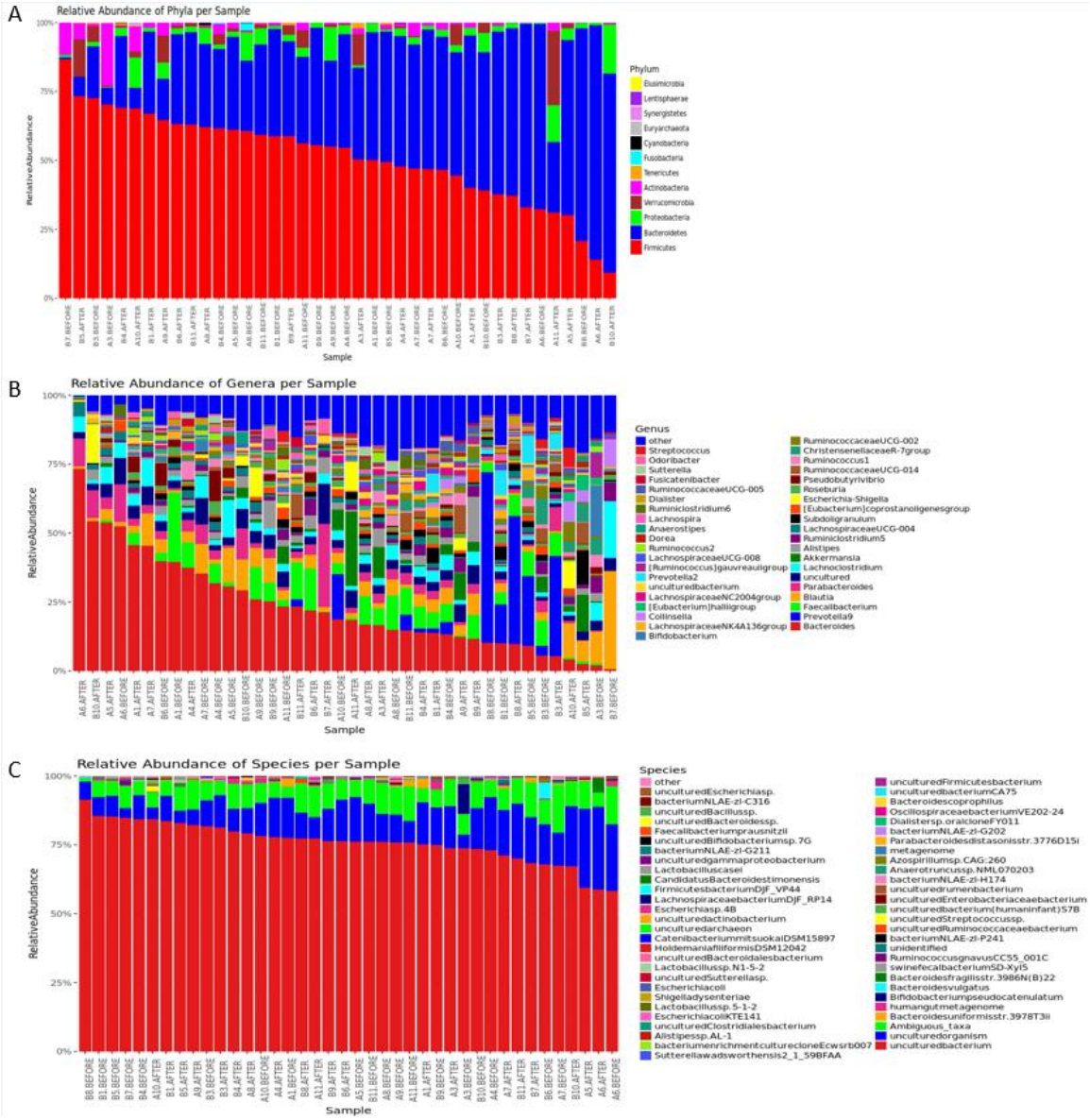

Figure 2-S3. Relative abundance of phyla **(S3A)**, genera **(S3B)** and species **(S3C)** per individual sample. *A* and *B* before sample numbers indicate the synbiotic and placebo groups, respectively.

The data obtained confirmed that *Firmicutes* and *Bacteroidetes* were the two most abundant bacterial phyla in the gut (see **Figure 2-1A**) and *Bacteroides* was the most

abundant genus (see **Figure 2-1B**). The synbiotic supplementation induced statistically significant alterations in the composition of the gut microbiota at the end of trial, as compared with the placebo group (**Figure 2-2**). All data were remained significant after adjusting for multiple testing (supplemental **Table 2-S5**). At the phylum level, increases in relative abundance of *Cyanobacteria, Euryarchaeota, Fusobacteria,* and *Lentisphaerae* were observed following synbiotic intervention. At the genus level, relative abundance of *Ruminococcus, Bifidobacterium, Sutterella, Tyzzerella, Eisenbergiella, Eubacterium, Eggerthella, Methanobrevibacter, Lachnospiraceae, Edwardsiella, Lactobacillus, Allobaculum, Enterococcus, Hydrogenoanaerobacterium, Coprococcus,* and *Butyricimonas* were significantly higher. The relative abundance of *Ruminococcaceae, Prevotella, Gardnerella, Turicibacter,* and *Megasphaera* at the end of trial was significantly lower in the synbiotic group, as compared with the placebo group. These results indicate that the synbiotic supplement used in the study modified relative abundance of gut bacteria, some of which can be associated with health benefits (particularly, significantly increased abundance of *Bifidobacterium* and *Lactobacillus*).

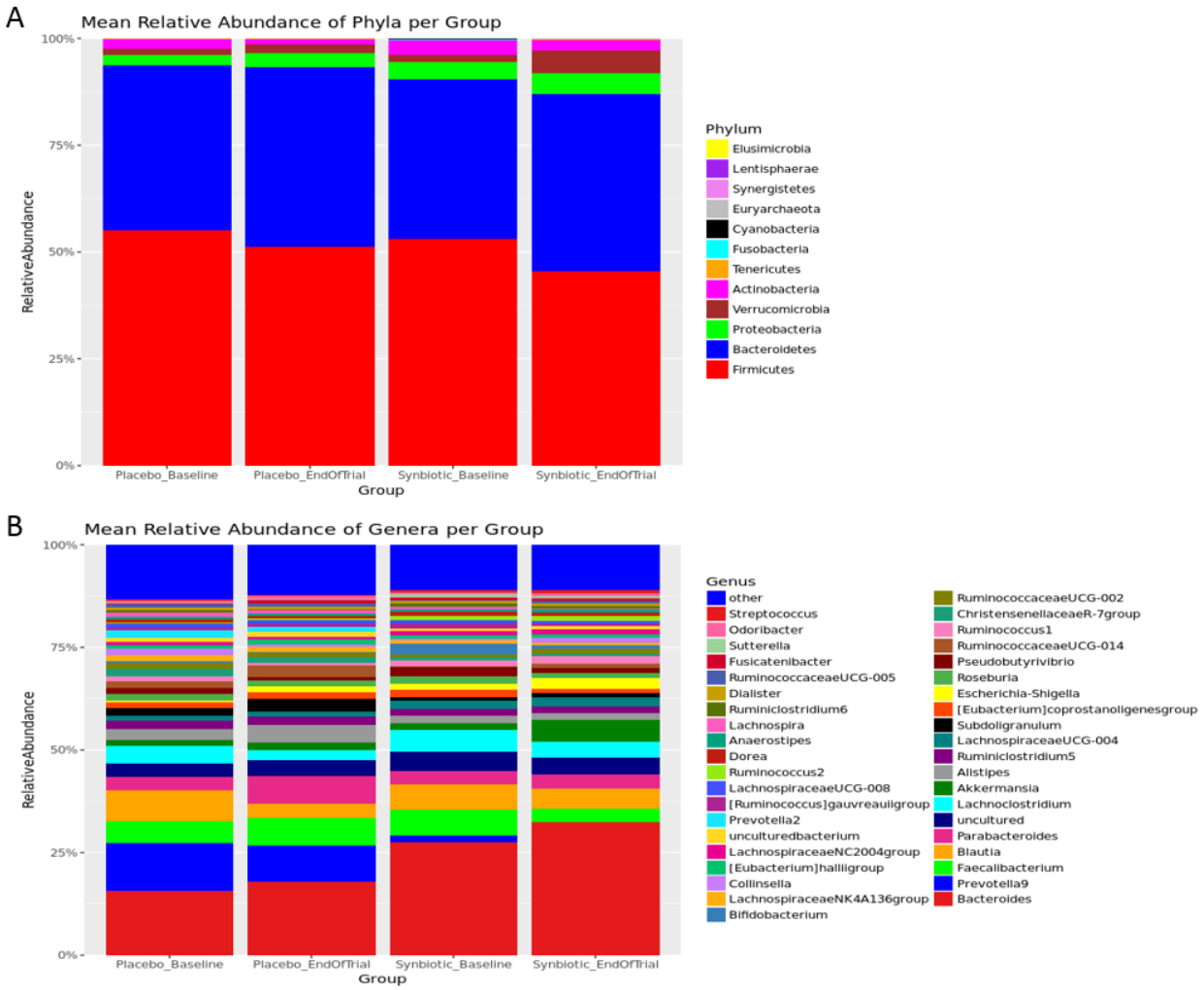

Figure 2-1. Mean relative abundance (%) of phyla (A) and genera (B) by the treatment groups and time points.

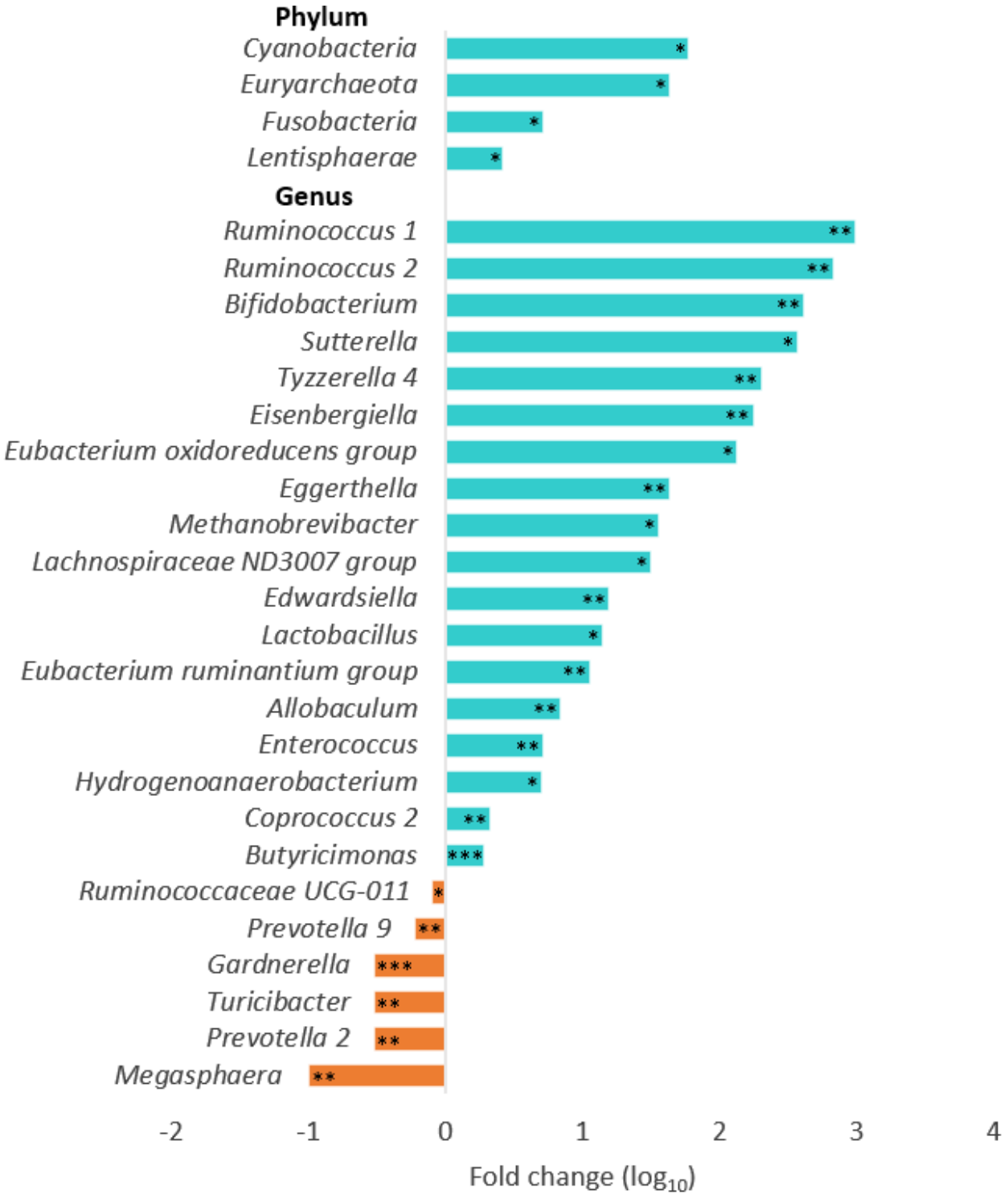
Phylum
Cyanobacteria
Euryarchaeota
Fusobacteria
Lentisphaerae
Genus
Ruminococcus 1
Ruminococcus 2
Bifidobacterium
Sutterella
Tyzzerella 4
Eisenbergiella
Eubacterium oxidoreducens group
Eggerthella
Methanobrevibacter
Lachnospiraceae ND3007 group
Edwardsiella
Lactobacillus
Eubacterium ruminantium group
Allobaculum
Enterococcus
Hydrogenoanaerobacterium
Coprococcus 2
Butyricimonas
Ruminococcaceae UCG-011
Prevotella 9
Gardnerella
Turicibacter
Prevotella 2
Megasphaera
-2
-1
0
1
2
3
4
Fold change (log10)

Figure 2-2. Changes in the relative microbial abundance in the gut after synbiotic intervention. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared with the placebo group at the end of trial.

Gut microbiota	***p*-value**
Megasphaera	0.003
Prevotella 2	0.001
Turicibacter	0.004
Gardnerella	0.0001
Prevotella 9	0.001
Ruminococcaceae UCG-011	0.019
Butyricimonas	0.0001
Coprococcus 2	0.007
Hydrogenoanaerobacterium	0.018
Enterococcus	0.001
Allobaculum	0.002
Eubacterium ruminantium group	0.008
Lactobacillus	0.041
Edwardsiella	0.001
Lachnospiraceae ND3007 group	0.012
Methanobrevibacter	0.015
Eggerthella	0.001
Eubacterium oxidoreducens group	0.011
Eisenbergiella	0.009
Tyzzerella 4	0.005
Sutterella	0.013
Bifidobacterium	0.004
Ruminococcus 2	0.003

Ruminococcus 1	0.002
Lentisphaerae	0.037
Fusobacteria	0.037
Euryarchaeota	0.022
Cyanobacteria	0.047

Table 2-S5. Bonferroni adjusted P-values. The Bonferroni correction sets the significance cut-off at α/n. With 28 tests (gut microbiota, n=28) and (α= 0.05), we'd only reject a null hypothesis if the p-value is less than 0.0017.

Alpha diversity metrics were used to measure species richness and evenness (similar abundance) in the groups (**Figure 2-3** and **Table 2-2**). The number of OTUs, the Chao1 estimator (a measure of community richness), and the Shannon Index (a measure of richness and evenness or entropy) were calculated. Data analysis showed that there were no significant differences in alpha diversity metrics between treatment groups and time points (**Figure 2-3B**). Shannon index pointed to a decreasing trend in microbial diversity at the end of trial in both the placebo and synbiotic groups (**Figure 2-3C**). This data suggests that the observed decrease in microbial diversity in the placebo and synbiotic groups at the end of trial implies involvement of other factors, probably, the effect of the high-protein, low-carbohydrate, energy-restricted diet used in this weight loss program.

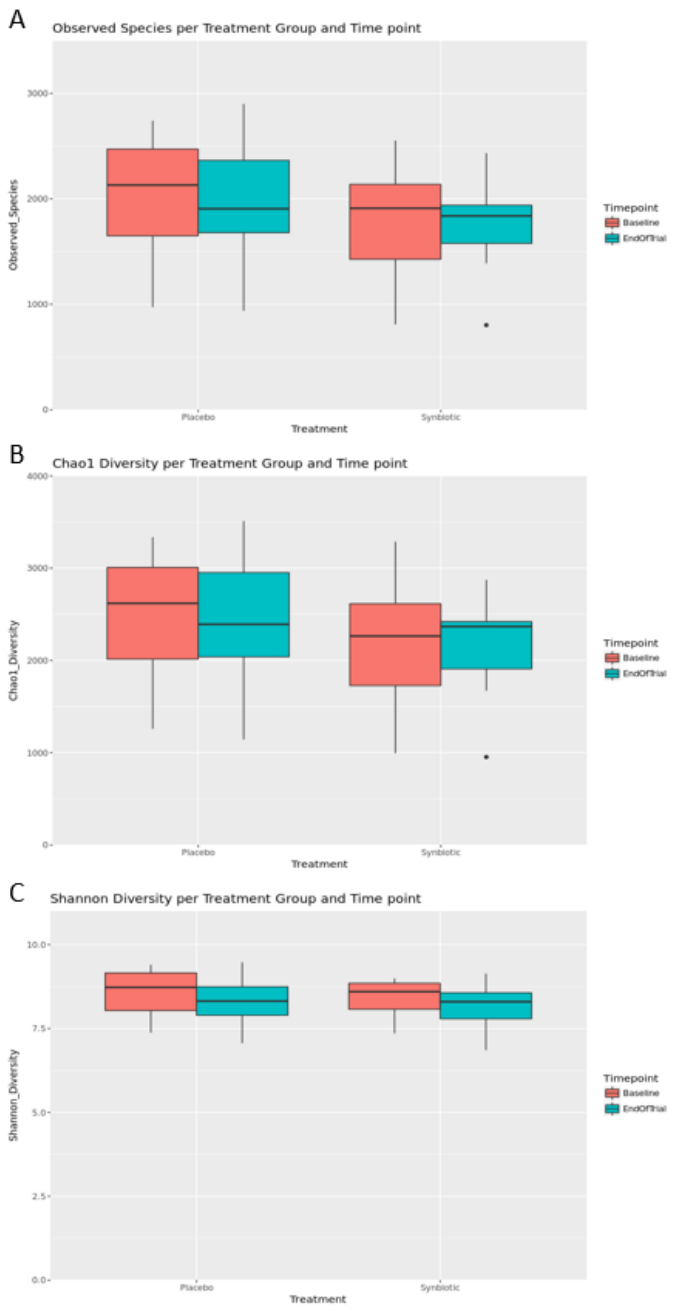

Figure 2-3. Observed species *(A)*, Chao1 diversity *(B)* and Shannon diversity *(C)* plotted by the treatment group and time point. The box spans the first and third quartiles. A horizontal line marks the median and the whiskers represent ±1.5-times the interquartile range. Outliers (panels A and B) are marked as individual points. Significant differences between groups were determined using the estimated marginal means analysis applied to

linear mixed model, which was built with alpha diversity as the response variable, the treatment group and time points as predictor variables, and subject number as a random variable.

Groups		**Shannon Index**		**Chao1 Diversity**		**Observed Species**	
Within	Between	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value
Baseline	Placebo - Synbiotic	0.144	0.643	295.0	0.359	222.0	0.388
End of Trial	Placebo - Synbiotic	0.145	0.641	205.4	0.521	180.9	0.481
Placebo	Baseline - End of Trial	0.208	0.208	76.94	0.577	76.20	0.460
Synbiotic	Baseline - End of Trial	0.209	0.206	-12.65	0.927	35.10	0.732

Table 2-2. Measuring statistically differences in alpha diversity between groups. Three alpha diversity metrics were used (Shannon Index, Chao1 Estimator, and Observed Species/OTUs). Significant differences between groups were determined using the estimated marginal means analysis applied to linear mixed model, which was built with alpha diversity as the response variable, the treatment group and time points as predictor variables, and subject number as a random variable.

Beta diversity metrics were used to compare differences in the community composition of two different samples. Bray-Curtis dissimilarity was used to compare the abundance of each OTU between two samples to give a metric between 0 and 1; weighted UniFrac distance, which is a dissimilarity metric that uses the phylogenetic distribution of the OTUs in a sample together with the abundance of those OTUs to

measure the distance between two samples; and unweighted UniFrac distance, which also measures the phylogenetic distribution of the OTUs in a sample, but relies only on presence/absence data instead of abundance data [158]. An assessment of the distances within and between time points and groups did not reveal significant changes in the community structure (**Table 2-3**).

Groups		**Bray-Curtis Dissimilarity**		**Weighted UniFrac**		**Unweighted UniFrac**	
Within	Between	F-model	*p*-value	F-model	*p*-value	F-model	*p*-value
Baseline	Placebo - Synbiotic	1.393	0.133	0.840	0.516	1.155	0.232
End of Trial	Placebo - Synbiotic	1.389	0.158	0.923	0.379	1.038	0.325
Placebo	Baseline - End of Trial	0.376	0.996	0.389	0.932	0.351	1.000
Synbiotic	Baseline - End of trial	0.431	0.983	0.305	0.958	0.392	1.000

Table 2-3. Measuring statistical significant of beta diversity differences between groups using Permutational Multivariate Analysis of Variance (PerMANOVA) on models with beta diversity as the response variable, and treatment group and time point as predictive variables. Three beta diversity metrics were used (Bray-Curtis, weighted UniFrac, and unweighted UniFrac).

To visually identify whether groups of samples cluster based on similarity to each other, PCoA plots were generated to highlight separation of groups of samples for unweighted UniFrac distance, weighted UniFrac distance, and Bray-Curtis dissimilarity distance (**Figure 2-4**). No statistically significant differences in microbial diversity

between or within the placebo and synbiotic group at the baseline and end of trial were observed.

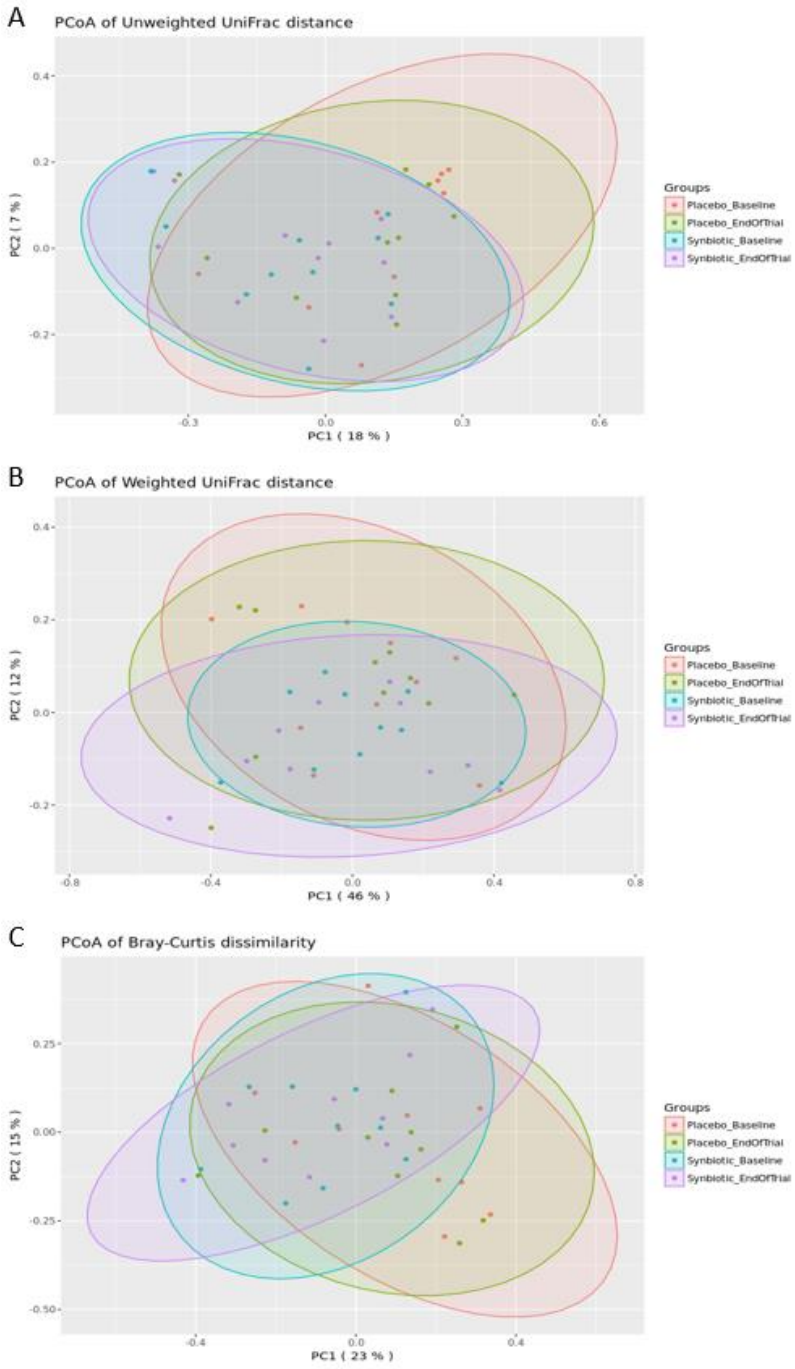

Figure 2-4. Principal Coordinates Analysis (PCoA) of unweighted UniFrac *(A)*, weighted UniFrac *(B)* and Bray-Curtis dissimilarity data *(C)*. Scatter plots show principal

coordinate 1 (PC1) vs. principal coordinate 2 (PC2) with percentages of variation explained by the components indicated. Points are colored by the treatment group and time point.

2.3.3. Associations between gut microbiota, body composition and metabolic parameters In order to explore associations between the gut microbial species and body composition and metabolic parameters, regression and correlation analyses were performed as described in the *Methods* section. Regression analysis to correlate relative microbial abundance of species present in the synbiotic supplement with body composition parameters and biomarkers of obesity found association between a decrease over time in blood glucose and an increase in *Lactobacillus* abundance in the synbiotic and placebo groups. In both groups combined, a mean decrease in HbA1C% (5.85%, see Table 1) was accompanied by a mean increase in *Lactobacillus* abundance (24.1-fold, see Fig. 2; $p = 0.044$). However (and somewhat paradoxically), a decrease over time in body mass, BMI, waist circumstance, and body fat mass was associated with a statistically significant decrease in *Bifidobacterium* abundance in both the placebo and synbiotic groups (**Table 2-4**).

Parameters	**Change**	**Gut microbiota**	**Change**	**P**
HbA1C%	↓ 5.85%	*Lactobacillus*	↑ 24.1- fold	0.044
Body mass (kg)	↓ 7.86%	*Bifidobacterium*	↓ 263.8- fold	0.052
BMI (kg/m^2)	↓ 7.98%	*Bifidobacterium*	↓ 263.8- fold	0.009
WC (cm)	↓ 5.90%	*Bifidobacterium*	↓ 263.8- fold	0.023
Body Fat Mass (kg)	↓ 7.89%	*Bifidobacterium*	↓ 263.8- fold	0.011

Table 2-4. Association between changes over time in (body composition and metabolic parameters) and changes in gut microbiota abundance in the synbiotic and placebo groups (both groups combined). BMI, Body Mass Index; WC, Waist Circumference; HbA1C, glycated hemoglobin. Data was generated by applying analysis of variance to a mixed linear model, built with the abundance of a given microbe as the response variable, and body composition, metabolic parameter, treatment groups and time points as the predictor variables, with subject number as random variable.

The Pearson's linear correlation test (**Figure 2-5**) did not indicate statistically significant associations between *Bifidobacterium* and *Lactobacillus* abundance and body composition parameters in the synbiotic group at the end of trial. A negatively correlated trend was observed between *Bifidobacterium* abundance and HbA1C levels in the synbiotic and placebo groups, whereas a positively correlated trend between *Bifidobacterium* abundance and, to a lesser extent, *Lactobacillus* abundance was observed with BMI, WC, and body fat mass in the synbiotic group. Interestingly, in the placebo group, *Lactobacillus* abundance was negatively correlated with body fat mass.

Cyanobacteria, *Sutterella*, *Butyricimonas*, and *Eubacterium ruminantium* abundance (which were increased following the synbiotic intervention) were significantly negatively correlated with body fat mass, and *Cyanobacteria* and *Sutterella* abundance was negatively correlated with body fat percentage. Additionally, *Butyricimonas* abundance positively correlated with BMC. *Eubacterium* abundance positively correlated with HbA1C percentage, whereas *Megasphaera* abundance (which was decreased after the synbiotic intervention) was negatively correlated with this marker. Positive correlations were found between *Coprococcus* abundance and body mass, BMI, and WC; *Lachnospiraceae* abundance and BMI, WC, and body fat mass; *Tyzzerella* and *Gardnerella* abundance and WC.

Figure 2-5. Heatmap of associations between gut microbiota, body composition and metabolic parameters in the placebo *(A)* and synbiotic groups *(B)* at the end of trial. *r* values were calculated using Pearson's linear correlation test; $^{*}p < 0.05$, $^{**}p < 0.01$. Pearson's *r* values below 0.30 or above -0.30 are not indicated. Red-brown color

indicates negative correlation, blue-green color -- positive correlation.Our data confirmed several previously reported associations [159-161], however, correlations found for *Lactobacillus* and *Bifidobacterium* were somewhat unexpected, although appear to be promising for associations with blood glucose levels. The results obtained support the conclusion that the synbiotic supplement used in this intervention trial modulated the microbiota by increasing abundance of the microbial genera associated with beneficial effects. Furthermore, these microbial changes may be associated with positive effects on metabolic parameters (blood glucose) in obesity.

2.4. Discussion

This study was a placebo-controlled intervention clinical trial designed to examine the effects of a combination of probiotic bacteria *L. acidophilus, B. lactis, B. longum, B. bifidum* and a prebiotic mixture of galactooligosaccharides on the human gut microbiota in relation to changes in body composition and metabolic biomarkers in obese human subjects enrolled on a weight loss program. The weight loss program was a high-protein, low-carbohydrate, energy-restricted eating plan. Previous limited studies conducted using *L. acidophilus* and *B. lactis* have found that these probiotic species can be associated with decreased body weight and body fat percentage [162], while prebiotic galactooligosaccharides have been shown to improve markers of metabolic syndrome and modulate the gut microbiota and immune function in overweight adults [153, 163, 164]. However, this study focused on evaluating the effects of synbiotic supplementation in obesity during weight loss intervention.

The study has confirmed that a high-protein, low-carbohydrate, restricted-energy diet can be effectively used for weight loss in obese individuals, but it also confirmed that

such a diet is associated with specific changes in gut microbiota. Previous evidence has shown that synbiotic supplementation contributes to altering microbial composition, resulting in benefits to weight loss and maintenance [165]. In the current study supplementation resulted in microbial changes that have frequently been associated with benefits to host health. However, how these changes relate to metabolic health remains to be confirmed. Furthermore, the microbial breakdown of proteins within the large intestine has been associated with the production of genotoxic and cancer associated metabolites, e.g. *N*- nitroso compounds and ammonia [144]. As such, altering the gut community to one less proteolytic through the introduction of a synbiotic could be of benefit to the host.

A combination of the four strains of *Bifidobacterium* and *Lactobacillus acidophilus* in the synbiotic supplement resulted in a significant increase in abundance of these probiotic genera in the gut after a 3-month intervention. *Bifidobacterium* is largely considered a positive member of the microbial community and furthermore, there has been some association with anti-obesity effects [162, 166, 167]. In addition to this, further modulation of the microbiota was observed, for example, *Prevotella* and *Gardnerella* genera were significantly decreased after the synbiotic intervention (see Figure 2-2). Previous studies have reported that these genera are associated with chronic inflammatory conditions and positively correlated with obesity [168-170]. Therefore, the reduction in these genera could help to modulate the balance to improve metabolism within the host. Special caution is warranted when analyzing the data referring to *Prevotella*, a complex genus linked both to health and disease and maybe influenced by race/ethnicity [171]. However, statistically significant differences in the community

composition of gut microbiota between groups (synbiotic vs. placebo) and time points (end vs. beginning of trial) using parameters of alpha-diversity (see Table 2-2) and beta-diversity (see Table 2-3) were not observed. Our data is compatible with a recent study that did not found a relationship between severe caloric restriction and changes in alpha-diversity[172]. In addition, correlation and regression analyses did not indicate statistically significant or apparently beneficial associations between species contained in the synbiotic supplement (*Bifidobacterium* and *Lactobacillus*) and body composition parameters, including at the end of synbiotic intervention (see Figure 2-5B). Interestingly, the changes over time in body mass, BMI, waist circumstance, and body fat mass demonstrated a positive correlation trend with *Bifidobacterium* abundance in the synbiotic group, while changes in body fat mass were negatively correlated with *Lactobacillus* abundance in the placebo group. However, positive associations between relative abundance of *Bifidobacterium* and several body composition parameters appear to point to the unfavorable role of these bacteria in promoting weight loss, although any potential benefits of this genera could be masked by the high-protein diet used in the study. High protein intake induces proteolytic fermentation in the gut with synthesis of compounds that have been implicated in the development of obesity and metabolic syndrome and modulating the gut microbiota [140-142, 173] and the production of toxic metabolites [174]. Several studies have also found that an increase in *Bifidobacterium* and *Lactobacillus* abundance is correlated with both pro- and anti-obesity effects in obese human subjects [175, 176], thus complicating the interpretation of the results. Individual differences in energy extraction may contribute to explain the observed differences [177]. Additionally, Bifidobacterium have been linked with improved barrier function in

overweight individuals, thus adding a potential beneficial mechanism of action [81]. Therefore, more studies are needed to fully understand the observed divergences and correctly identify human subpopulations susceptible to benefit from synbiotic intervention.

Regression analysis performed to correlate microbial abundance of species contained in the synbiotic supplement with biomarkers of obesity found a novel significant association between a decrease over time in HbA1C percentage and an increase in Lactobacillus abundance, particularly in the synbiotic group. This is an important observation because it demonstrates the beneficial effect of increasing *Lactobacillus* abundance on potentially reducing blood glucose levels. Negative associations between *Megasphaera* abundance and *Eubacterium ruminantium* abundance with HbA1C levels were observed in the synbiotic group at the end of trial. *Eubacterium ruminantium* are xylanolytic bacteria (i.e., producing xylanase following dietary fiber fermentation) and Megasphaera bacteria utilize lactate [178], which can underlie potential relationship of these species to decreasing blood glucose levels [179, 180]. However, within the trial following the synbiotic a decrease in *Megasphera* was observed, an increase in *Eubacterium ruminantium*. This could imply that the synbiotic intervention and associated microbial changes could be linked to maintaining a normal blood glucose levels in obesity.

It should also be considered that the microbial shifts observed in this study may be associated with a positive impact on microbial fermentation within the large intestine. Whilst the microbial changes observed following synbiotic intervention included an increase in *Ruminococcus*, a genera known to produce butyrate. Butyrate is a short chain

fatty acid that provides and energy source for the colonocytes, as a histone deacetylase inhibitor this SCFA is linked to anti-cancer effects, hence could offer protection against some of the metabolites that are produced within a high protein diet. SCFA's have also recently been associated with protection against type 1 diabetes [181]. In addition to this lactobacilli and bifidobacteria are associated with positive effects of colonic health and following synbiotic intervention have been associated with reducing fecal water genotoxicty, which is considered a biomarker for colon cancer [182]. Therefore, whilst within the weight-loss diet employed the synbiotic treatment may have had a limited impact on the weight-loss parameters, it is possible that the changes in the microbiota could help to reduce detriments associated with a high protein diet.

It is important to emphasize that the present study was a randomized, placebo-controlled intervention clinical trial and that analysis of the community composition of the gut microbiota between the treatment groups and time points was performed using comprehensive microbiome analysis, including alpha- and beta-diversity metrics and multivariate analysis of variance. The design of the study has allowed us to detect important novel associations between composition of the gut microbiota and metabolic parameters in obesity in the relatively limited number of participants in this clinical trial.

The results obtained and bioinformatic analysis support the conclusion that weight loss in human subjects participating in a high-protein, low-carbohydrate, energy-restricted eating weight loss program is accompanied by changes in gut microbiota that can be associated with increased genotoxicity [144]. The synbiotic used in this study modulated the human gut microbiota by increasing abundance of the microbial species that can be considered to be of benefit to their host; it was also associated with positive

effects on metabolic parameters in obesity. Thus, the addition of synbiotic supplements to weight reduction diets may aid against negative microbial changes associated with high protein diets and weight loss.

Author Contributions: INS and TA conducted the research, analyzed the data and wrote the manuscript; EH designed the research and obtained funding; INS supervised the research project; EH and GW contributed to review of the manuscript. All authors provided critical feedback and helped shape the research, analysis, and read and approved the final manuscript.

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Conflict of Interest: The authors declare no conflict of interest.

CHAPTER 3. Effects of probiotic dairy products on gut microbiota and body weight in mice

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Abstract: Targeting gut microbiota with probiotics has recently emerged as a promising nutritional approach for the prevention of obesity and metabolic syndrome. Cultured dairy products can be effectively employed for the delivery of probiotics to the gut as well as for the support of growth and survival of probiotic bacteria. The purpose of this study was to characterize the effects of probiotic-enriched pasteurized milks and dairy products (Greek-style yogurt and cheese) of different origins (cow, goat, and camel) on the taxonomic composition of the mouse gut microbiota and body weight. Mice were fed standard low fat, plant polysaccharide-rich (LF/PP) diet supplemented with the probiotic-enriched dairy products for 5 weeks. Next generation DNA sequencing from mouse fecal samples was used to obtained data on the bacterial relative abundance. Mice fed a diet supplemented with camel milk demonstrated a decrease in body weight gain as compared

with mice fed LF/PP diet. This was accompanied by characteristic changes in the gut microbiota, which included an increase in relative abundance of order *Clostridiales* and genus *Anaerostipes*. Mice fed diet supplemented with the probiotic cheese exhibited a decreasing trend in body weight gain, accompanied by an increase in the relative abundance of order *Clostridiales,* family *Ruminococcaceae*, and family *Lachnospiraceae*. The results obtained and their bioinformatics analysis support the conclusion that camel milk and probiotic cheese induce changes in the mouse gut microbiota, which can be characterized as potentially health beneficial compared to changes associated with standard diet or diets supplemented with cow milk, goat milk, and yogurt. These findings imply that dairy products are effective for the delivery and supporting growth of probiotics bacteria in the gut and, thus, may contribute to maintaining healthy body weight.

Keywords: gut microbiota; cultured dairy products; probiotic-enriched dairy products; body weight

3.1. Introduction

Over the last decade an ever growing body of evidence has established the gut microbiota as one of the most important determinants of metabolic syndrome [183-185]. Importantly, the gut microbiota composition is modulated by several genetic and environmental factors, including diet among the most important factors [186-188]. Therefore, manipulation of the gut microbiota through dietary intervention and probiotic supplements can be a promising strategy for the prevention and treatment of metabolic syndrome and obesity. As a consequence, worldwide sales of probiotics have more than doubled in the last decade alone [114]. Probiotic bacteria can be delivered to the gut as dietary supplements or in foods, including dairy products, such as yogurt or kefir [115, 116].

More than 6 billion people worldwide consume milk and dairy products [189]. Milk provides 11 to 14 percent of dietary energy supply in Europe, Oceania and the Americas [190]. Recent studies have found an inverse relation between consumption of dairy products and metabolic syndrome and, probably, obesity [191, 192].

We hypothesized that cultured dairy products such as cheese and yogurt can be an ideal vector for the delivery of probiotics to the gut because of its nutritional value, acidic nature, and long shelf-life. Cheese is a suitable matrix for transferring probiotic bacteria into the gastrointestinal tract due to the high buffering capacity of the milk proteins which can protect the bacterial cells during transit. High fat content and dense structure of cheese protect bacteria against acidic environment of the gastrointestinal tract [117-119]. While several studies have shown an inverse association between dairy products and metabolic syndrome and obesity [120-122], studies that comprehensively analyze the

effects of different milk sources (cow, goat and camel) and products (milk, yogurt, probiotic yogurt, cheese, and probiotic cheese) on the gut microbiota composition and body weight are not available.

The objective of this study was to characterize the effect of probiotic-enriched pasteurized milk, Greek-style yogurt, and cottage cheese made from milk of different origins (cow, goat, and camel) on the taxonomic composition of the mouse gut microbiota and body weight. A well-established *Clostridium butyricum* [193, 194] was used as probiotic in these studies. The results obtained and their bioinformatics analysis appear to support the conclusion that camel milk and the probiotic cow cheese induce changes the mouse gut microbiota, which are associated with the optimal weight gain in growing mice.

3.2. Materials and Methods

3.2.1. Animals and diets

Female 6-8 weeks old C57BL/6 mice weighing 14-16 g, were purchased from Jackson Laboratory (Bar Harbor, ME). Upon arrival, they were randomly grouped and kept in specific pathogen free (SPF) conditions in individually ventilated cages with sterile bedding at 24-26°C, relative humidity 50 ± 10% and a 12-h light/dark cycle. Mice were fed *ad libitum* standard low fat, plant polysaccharide-rich (LF/PP) diet 5001 (LabDiet, St. Louis, MO) supplemented daily with the dairy products for 5 weeks. The control group was fed standard LF/PP diet without dairy supplementation. Three groups were supplemented with pasteurized cow milk (DairyPure, Dallas, TX, USA), goat milk (Meyenberg, Turlock, CA, USA) or camel milk (Desert Farms, Santa Monica, CA). Four

groups were supplemented with cow yogurt or goat yogurt (with and without probiotics). Finally, two groups were fed diet supplemented with either a regular cow cheese or a probiotic cow cheese. There were 5 animals in each experimental group. The amount of daily supplementations was 1 mL of dairy product per mouse per day. Body weight was measured weekly using top-loading balances with 0.01 g precision (OHAUS SPX222., Pine Brook, NJ). The experiments were approved by the South Dakota State University Institutional Care and Use Committee (Approval number: 16-024A).

3.2.2. Manufacture of Cultured Dairy Products

The probiotic cheese and probiotic yogurt were manufactured at the Health and Nutritional Sciences Department facilities utilizing the probiotic strains grown in our laboratory. The manufacture process for Greek-style yogurt and cottage cheese is schematized in **Figure 3-1,** following the procedure described elsewhere [195, 196]. One liter of yogurt and cottage cheese were produced weekly.

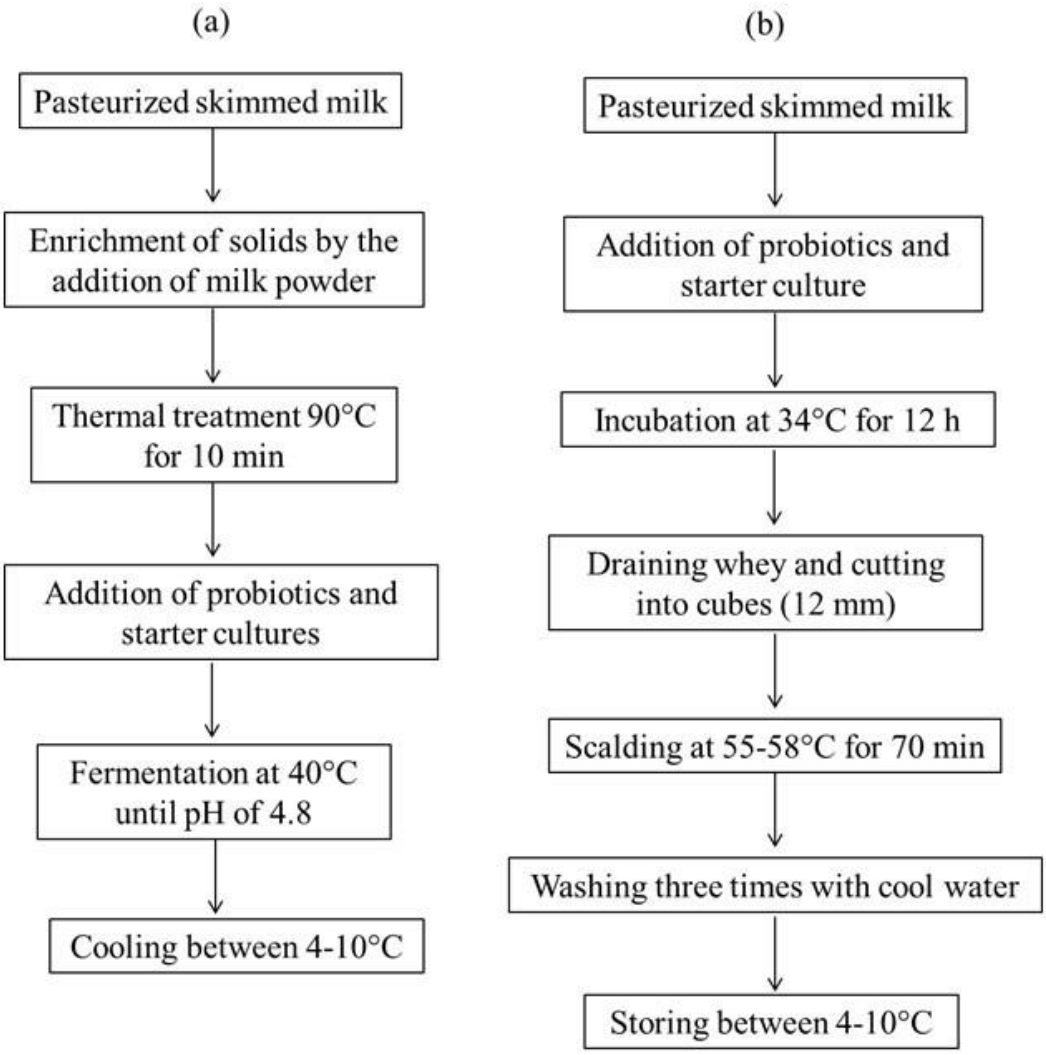

Figure 3-1. Schematic diagram for the manufacture of cultured dairy products: (a) Greek-style yogurt and (b) cottage cheese.

3.2.3. Microbial DNA Extraction and the 16S rRNA Gene Sequencing

Fecal samples from mice were obtained at the end of experimental study by taking individual mice out of their cage and gathering a stool sample in a 2 mL tube. All fecal samples were immediately frozen at -80°C. DNA was extracted from fecal sample

by using the microbial DNA extraction method (ZR Fecal DNA MiniPrep™) as described by [197]. Libraries and sequencing were performed at the South Dakota State University Genomics Sequencing Facility. Amplification of the V3-V4 amplicon (460bp) of the 16S RNA gene was achieved using primers described in Klindworth et al. [198]: 16SAmpliconPCRForwardPrimer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16SAmpliconPCRReversePrimer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. These primers include the Illumina overhang adapter sequences in their 5' ends. Amplification was carried out using KAPA HiFi HotStart Ready Mix (KAPABiosystems. Wilminton, MA) using the following polymerase chain reaction (PCR) thermal profile: 3min @ 95°C, 25 cycles (30sec @ 95°C, 30 sec @ 55°C, 30 sec @ 72°C), 5 min @72°C. PCR clean-up was done with a SMARTer Apollo system (Takara Inc. Mountain View, CA) using AMPure XP beads (Beckman Coulter. Indianapolis, IN). A second round of PCR amplification was carried out to introduce individual Nextera XT (Illumina Inc. San Diego, CA) indices in each library. This second amplification was carried out using KAPA HiFi HotStart Ready Mix (KAPABiosystems. Wilminton, MA) using the following PCR thermal profile: 3min @ 95°C, 8 cycles (30sec @ 95°C, 30 sec @ 55°C, 30 sec @ 72°C), 5 min @72°C; PCR clean-up was done the same way as described for the first PCR round. Sequencing was carried out in an Illumina MiSeq using vs 3 chemistry (3x300 bp).

3.2.4 Bioinformatics and Statistical analyses

All bioinformatics analyses were performed using the Microbial Genomics Module in CLC Genomics Workbench vs 12.0 (Qiagen). After trimming adaptors and lower quality reads (q=20) the pared reads were joined. Samples with less than 90,000 reads were removed from further analysis. Resulting operational taxonomic units (OTUs) were aligned to the GrainGenes database vs 13_5 at 97% similarity. Individual OTUs with less than 100 reads were removed from further analysis. All samples underwent taxonomic classification and were included in the complete OTU table (supplemental **Table 3-S2**). Statistical comparisons between group pairs were carried out using a Wald test; p values were False Discovery Rate (FDR) corrected.

For the body weight, differences between groups were determined by ANOVA, followed by the Fisher's multiple comparison procedure to identify differences in treatment means. The results were expressed as means ± S.E.M. Mean differences were considered significant at $p < 0.05$. Data were analyzed using SPSS 20.0.

3.3. Results

3.3.1. Effect of probiotic-enriched camel, cow, and goat milk on the gut microbiota profiles and body weight gain

The effect of different milks on body weight is shown in F**igure 3-2**. Mice were feed a standard LF/PP diet supplemented with the dairy products for 5 weeks. Mice in the group supplemented with cow milk demonstrated a small, but significant (p=0.004) weight gain when compared with the control group. However, mice fed diets supplemented with either goat or camel milk did not have a significant body weight gain compared to control animals.

Analysis of fecal samples for changes in the gut microbiota in different experimental groups is presented in **Figure 3-3**. Next generation gene sequencing of 16S rRNA (3x300bp PE V3 + V4) from fecal samples was used to obtain data on the bacterial relative abundance, which was analyzed at the level of order, family, and genus. The groups supplemented with different milks were then compared. A significant reduction in the relative abundance of order *Clostridiales* 191753 (p=0.01), *Clostridiales* 4455677 (p=0.02), family *Clostridiaceae* 268074 (p=0.02), and family *Peptostreptococcaceae* 276478 (p=0.01) was observed in the group supplementd with cow milk, while an increase of order *Clostridiales* 199532 (p=0.03), *Clostridiales* 271602 (p=0.001), and genus *Anaerostipes* 534926 (p=0.02) was demonstrated in the group supplemented with camel milk as compared with the group supplemented with cow milk **(Figure 3-3A)**. We also found that the relative abundance of family *Clostridiaceae* 268074 and *Peptostreptococcaceae* 276478 was significantly decreased in the camel milk group as compared with the goat milk group (p=0.003 and p=0.003), respectively **(Figure 3-3B)**. The relative abundance of order *Clostridiales* 271602 and genus *Anaerostipes* 534926 was significantly higher in the goat milk group compared with the cow milk group (p=0.03 and p=0.03), respectively, while a reduction in order *Clostridiales* 344198 (p=0.0003) was founded in animals supplemented with goat milk compared with the cow milk group **(Figure 3-3C)**.

A Venn diagram was established to clarify the overlap of OTUs (enriched for certain OTUs) between the camel, cow, and goat milk supplemented groups. The total significant richness in the dataset was 17 OTUs, with the most changes observed in the camel milk group vs. the cow milk group (9 total OTUs) **(Figure 3-4)**. We also observed

that the body weight gain in the camel milk group exhibited a decreasing trend at day 21 and 35 **(**see **Figure 3-2**). These findings suggest that the gut microbiota enrichment can be achieved via consumption of camel milk.

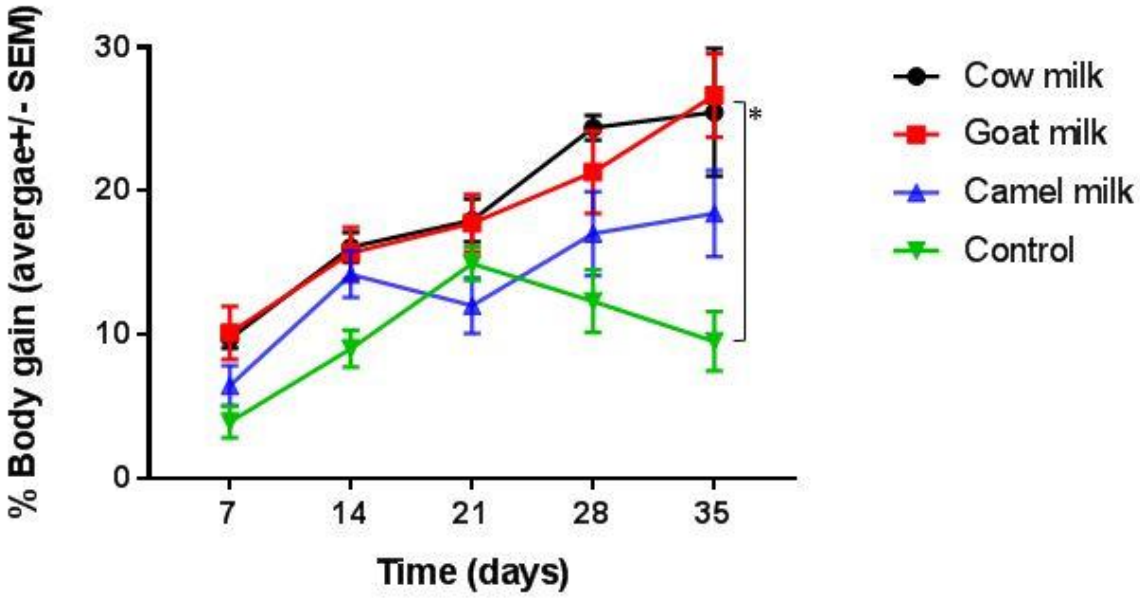

Figure 3-2. Percentage of body weight increase in mice fed diets supplemented with cow, goat, or camel milk. Data are presented as mean ± SEM. n=5 per group. $^{*}p < 0.05$,

as compared between the indicated groups and determined by ANOVA.

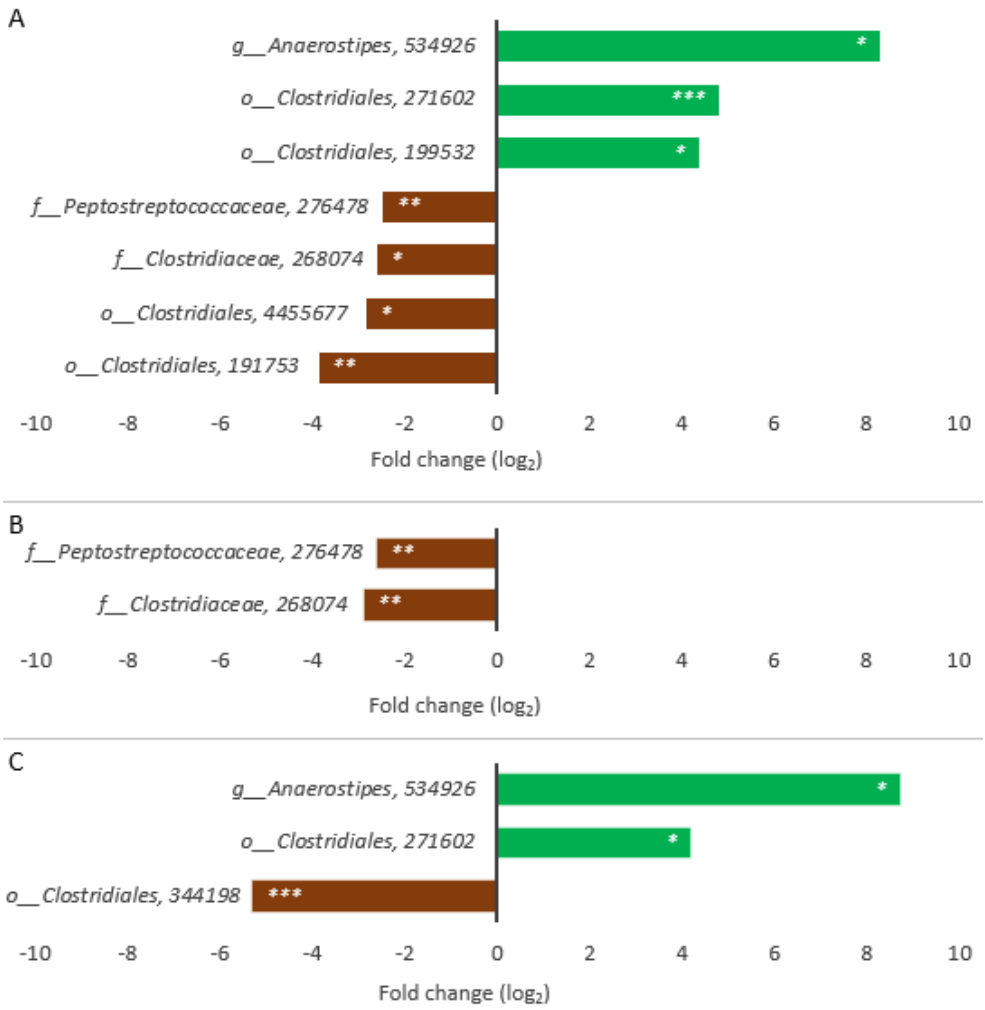

Figure 3-3. Relative abundance of the gut microbiota at the order (*o*), family (*f*), and genus (*g*) levels, as compared between the camel milk vs. cow milk groups (A), camel milk vs. goat milk groups (B), and goat milk vs. cow milk groups (C). FDR *p*-value are

$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

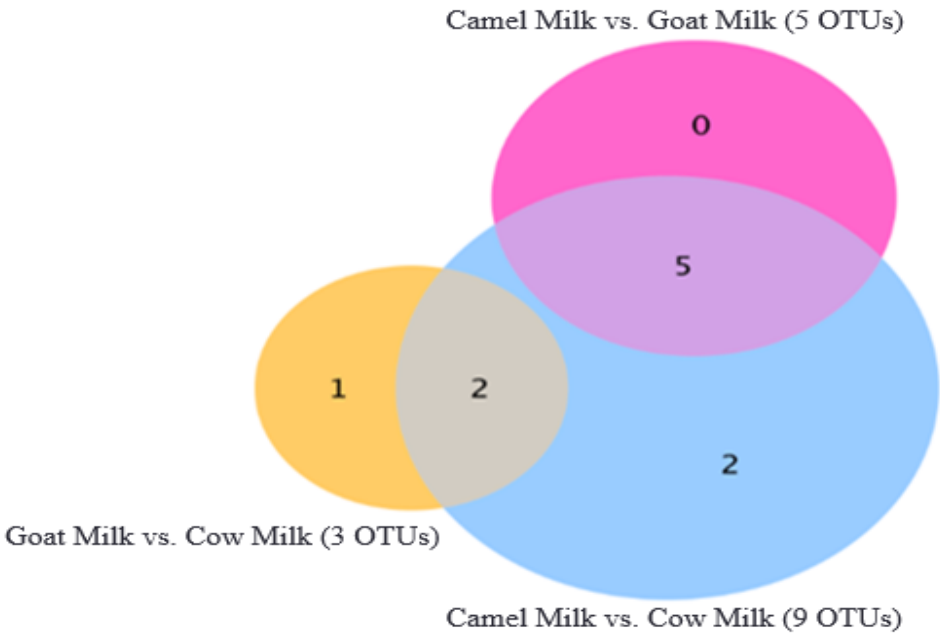

Figure 3-4. The overlap (enrichment) of microbial species (OTUs) between the camel, cow, and goat milk groups. The numbers indicate overlap in OTUs.

3.3.2. Effects of probiotic-enriched yogurt and cheese on the gut microbiota profiles and body weight gain

The effects of probiotic-enriched yogurt and cheese of different origins (cow and goat milk) on the gut microbiota and body weight were determined. Mice were fed LF/PP diet supplemented with the probiotic dairy products for 5 weeks. **Figure 3-5** shows that the body weight gain of the probiotic cow cheese group exhibited a decreasing trend at day 28 (p=0.08) and day 35 (p=0.07). The probiotic goat yogurt group demonstrated a significant body weight gain compared to the probiotic cow cheese group (p=0.01) and the cow cheese groups (p=0.03) at day 28. Individual body weight (g) and time (days) measurements of mice fed diet supplemented with dairy products, are presented in appendix Table 3-S1.

Fecal samples were analyzed for changes in the gut microbiota of mice in the experimental groups (**Figure 3-6**). The relative abundance of the order *Clostridiales,* family *Lachnospiraceae*, genus *SMB53,* and species *Ruminococcus gnavus* were significantly higher in the probiotic cow yogurt group vs. the cow yogurt group, while a decrease in relative abundance of family *Erysipelotrichaceae, Clostridiaceae,* genus *Oscillospira, Clostridium, Ruminococcus, Aerococcus,* species *R. gnavus* and *Clostridium perfringens* were observed comparing the same groups **(**see **Figure 3-6A)**.

Changes in the bacterial relative abundance were observed in comparisons between the probiotic goat yogurt group and the goat yogurt group. A significant decrease in abundance of order *Clostridiales* and family *Lachnospiraceae* were observed, while relative abundance of order *Clostridiales,* genus *Staphylococcus, Anaeroplasma, Anaerostipes,* and species *R. gnavus* was increased **(**see **Figure 3-6B)**.

In the probiotic cow cheese group vs. the cow cheese group **(Figure 3-7)**, the relative abundance of order *Clostridiales,* family *Ruminococcaceae, S24-7, Lachnospiraceae,* and species *R. gnavus* was significantly higher, whereas a decrease in relative abundance of order *Clostridiales,* family *Erysipelotrichaceae, Lachnospiraceae, S24-7,* genus *Coprococcus, Oscillospira,* and species *R. gnavus* was observed.

A Venn diagram was established to determine the overlap of OTUs between the yogurt and cheese groups **(Figure 3-8)**. The total significant richness in the dataset was found for 16 OTUs in the cow yogurt group **(Figure 3-8A)**, 13 OTUs in the goat yogurt group **(Figure 3-8B)**, and 30 OTUs in the cow cheese group. The most significant enrichment was found in the probiotic cow cheese group vs. the cow cheese group (30 total OTUs) **(Figure 3-8C)**. These findings suggest that the gut microbiota enrichment

can be linked to consumption of the probiotic cow cheese and imply that this cheese can be an efficient vector for the probiotic delivery.

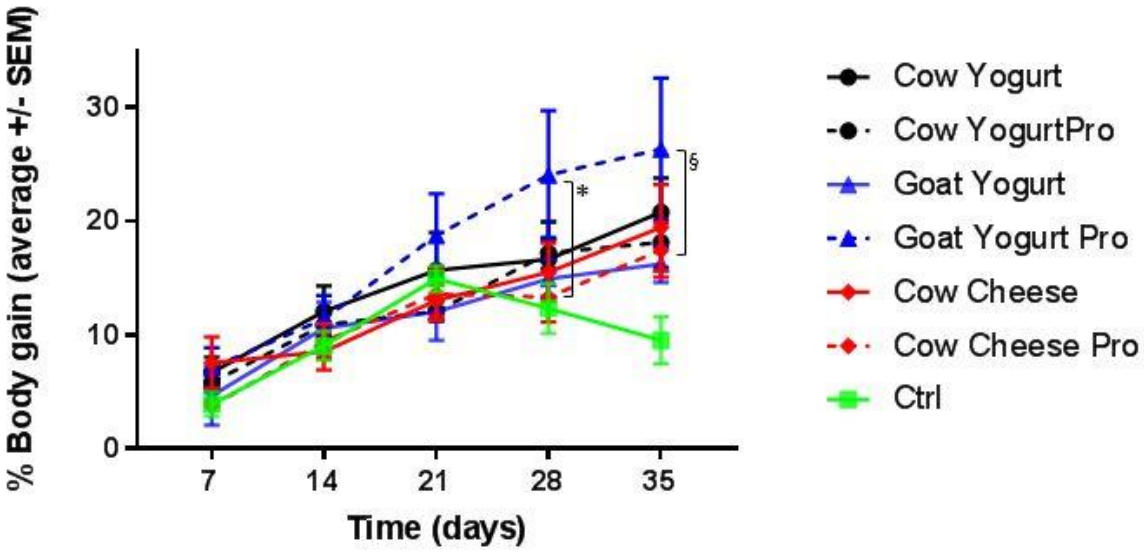

Figure 3-5. Percentage of body weight increase in mice fed diets supplemented with yogurt and cheese. Data are presented as mean ± SEM. n=5 per group. $^{*}p < 0.05$ and $^{\S}p < 0.08$ (approaching significance), as compared between the indicated groups and determined by ANOVA.

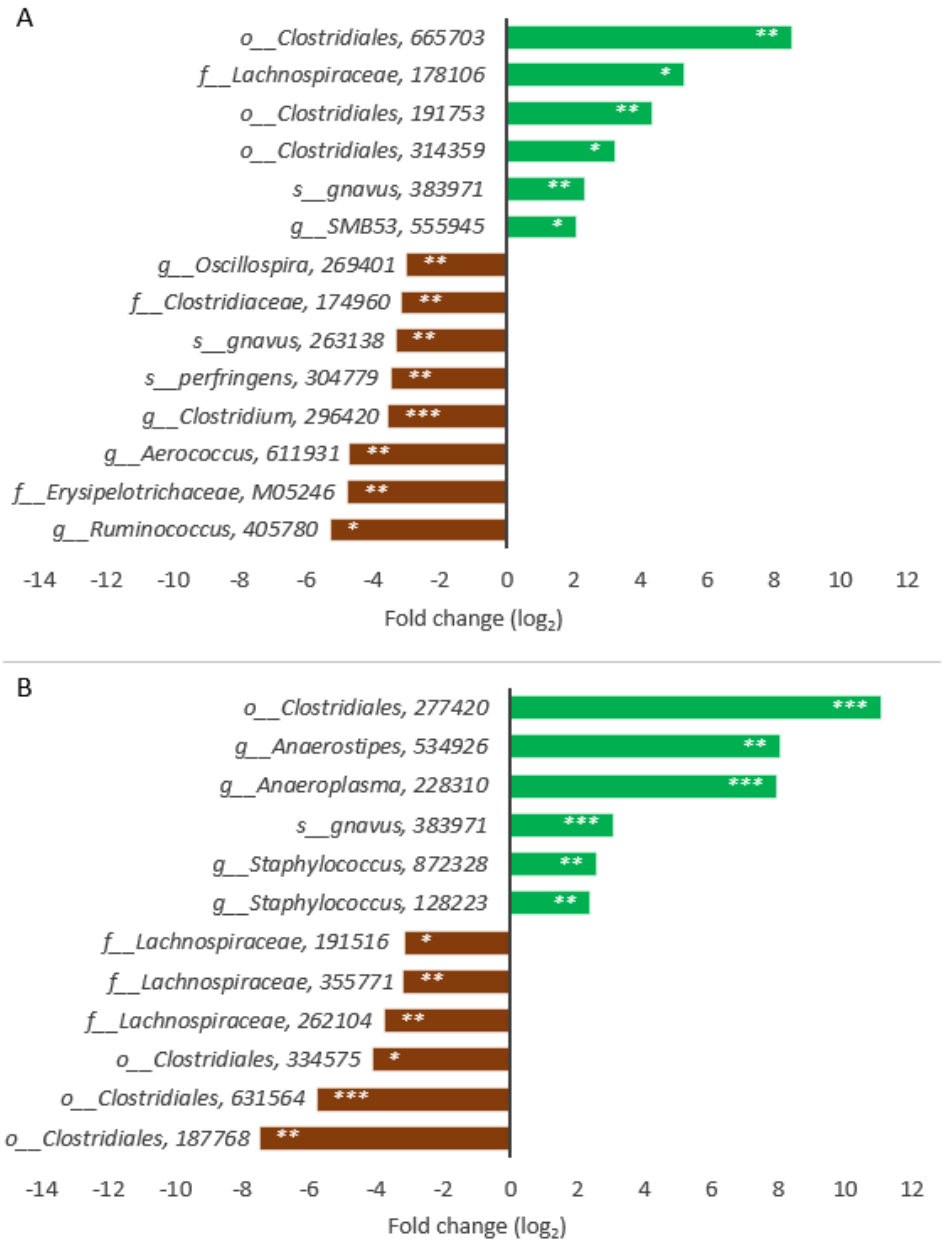

Figure 3-6. Relative abundance of the gut microbiota at the order (*o*), family (*f*), genus (*g*), and species levels (*s*), as compared between the probiotic cow yogurt group vs. the cow yogurt group (A) and the probiotic goat yogurt group vs. the goat yogurt group (B). FDR *p*-value are $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

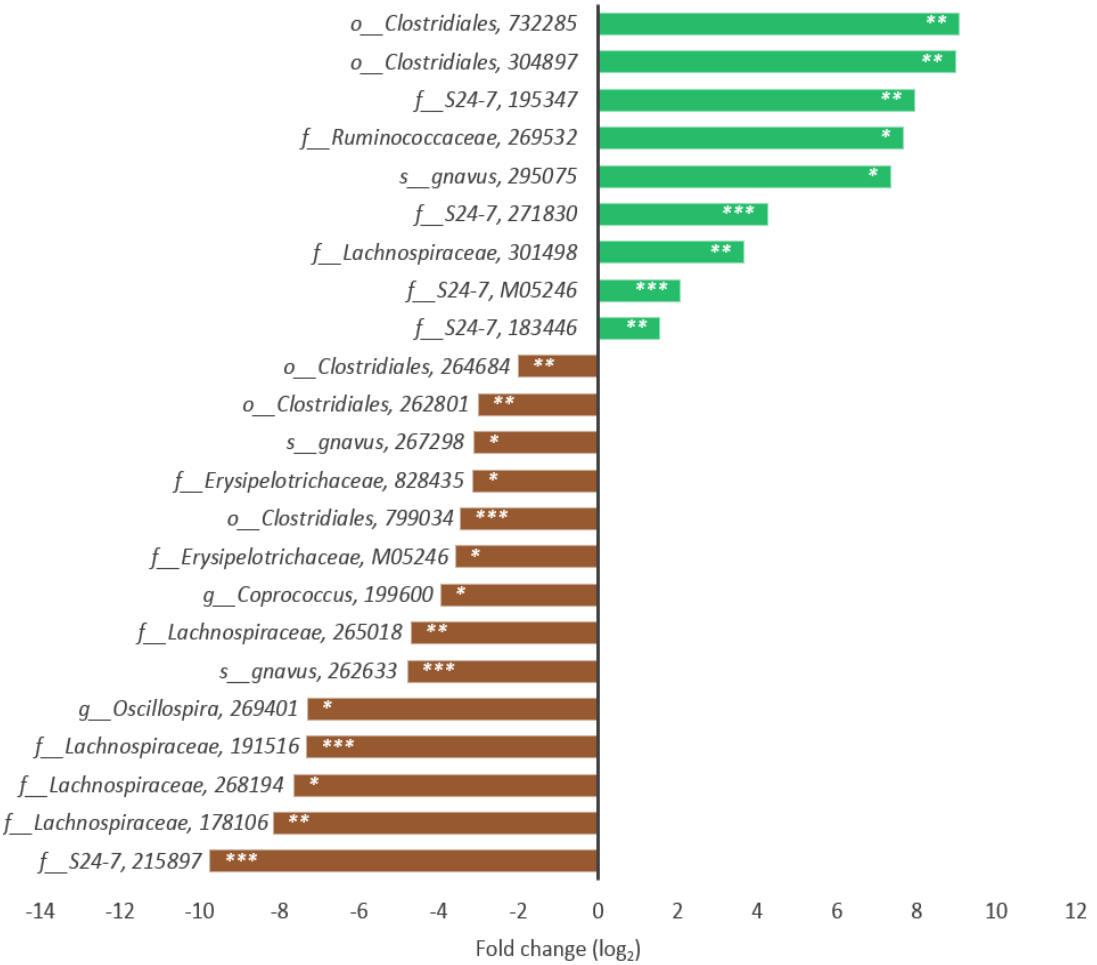

Figure 3-7. Relative abundance of the gut microbiota at the order (*o*), family (*f*), genus (*g*), and species levels (*s*), as compared between the probiotic cow cheese group vs. the

cow cheese group. FDR p-value are $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

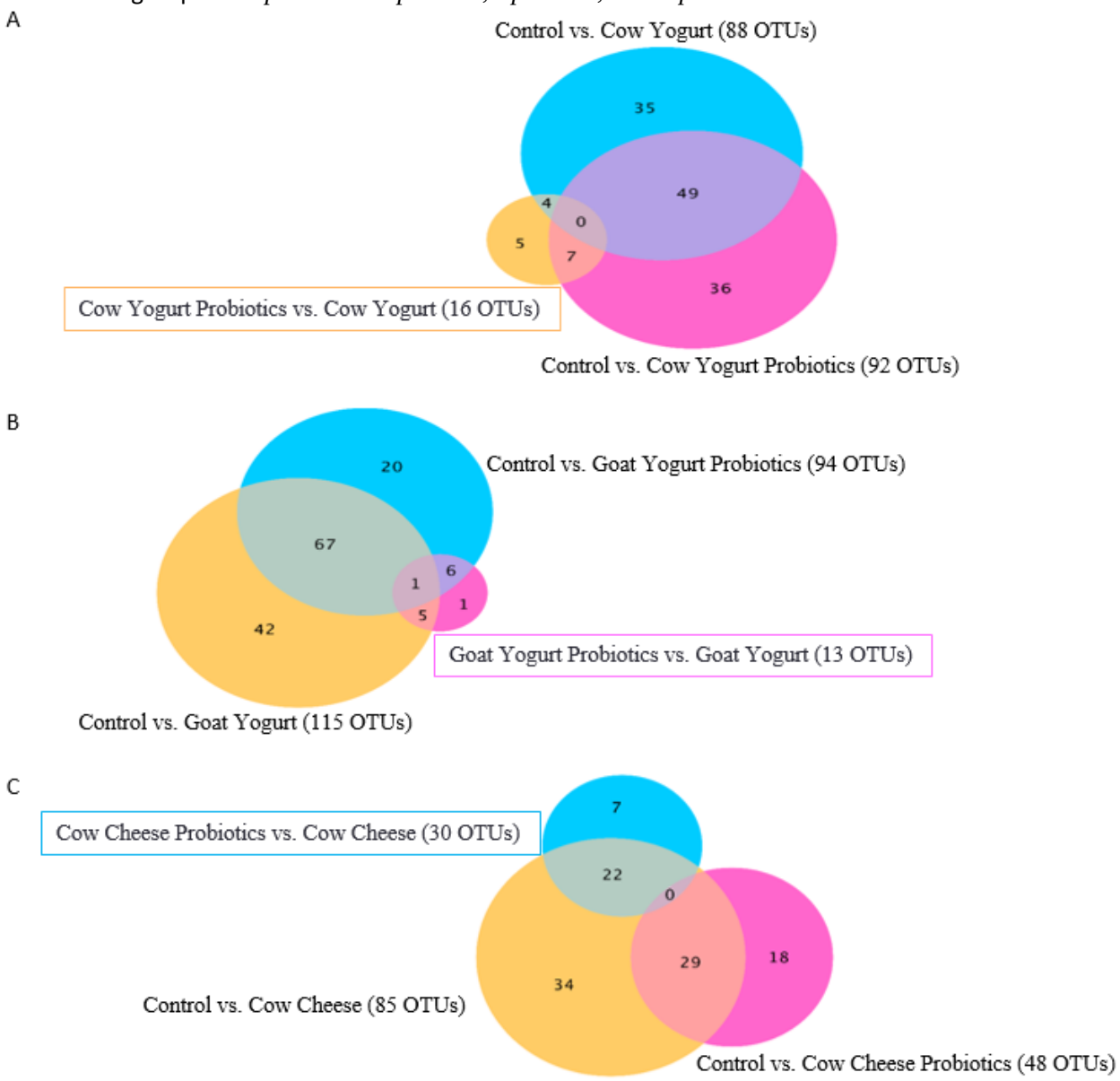

Figure 3-8. The overlap (enrichment) of microbial species (OTUs) between the yogurt and cheese supplemented groups. The numbers indicate overlap in OTUs.

3.4. Discussion

The present studies demonstrated that gut microbiota enrichment can be achieved via consumption of camel milk, as compared with cow and goat milk. In addition, a significant gut microbiota enrichment was observed in mice supplemented with the probiotic cow cheese. To the best of our knowledge, this is the first study that compared the effects of supplementation with dairy products originating from different animals on the gut microbiota composition.

The findings obtained indicate that, when compared with mice fed with cow milk, mice fed a diet supplemented with camel milk demonstrate a decrease in body weight gain (see Figure 3-2) accompanied by characteristic changes in the gut microbiota, including an increase in order *Clostridiales* and genus *Anaerostipes* (see Figure 3-3A). Importantly, these bacteria have been previously shown to produce short-chain fatty acids (SCFAs), a bacterial fermentation product of dietary fiber, recognized to have wide-ranging effects on maintenance of health and host physiology [199-201]. We also found a decreased abundance of *Peptostreptococcaceae* bacteria in the camel milk group. Importantly, species in this family are positively associated with obesity and inflammation [202-204].

The health benefits of camel milk may be partly explained by its functioning as a prebiotic. Camel milk has a high overall nutritional value and high heat stability in the process of preservation of raw milk by pasteurization [205, 206]. The induction of the SCFAs producing gut bacteria via consumption of camel milk may lead to development of nutritional strategy for weight loss and the prevention and treatment of obesity.

Surviving passage over the host's gastrointestinal tract is a critical event for any probiotic. In this regard, we observed that the total significant richness in the dataset were 16 OTUs for the probiotic cow yogurt group vs. the cow yogurt, 13 OTUs for the probiotic goat yogurt group vs. the goat yogurt group, and 30 OTUs for the probiotic cow cheese group vs. the cow cheese group (see Figure 3-8C). These results indicate that the probiotic supplement used in the study modified relative abundance of gut bacteria, some of which can be associated with health benefits (particularly, a significantly increased abundance of order *Clostridiales* due to the probiotic intervention; see Figure 3-7). Therefore, cheese appears to be a suitable matrix for transferring probiotic bacteria into the gastrointestinal tract, probably, due to the high buffering capacity of milk proteins which will protect the microbial cells during transit (dense product structure may also protect the bacteria against the acidic environment in the gastrointestinal tract [117-119]).

Clostridium butyricum supplementation triggered a shift in body weight gain with a significant decrease in the probiotic cow cheese group compared with the probiotic goat and cow yogurt groups (see Figure 3-5). We also found that the addition of this probiotic to cow cheese improves gut microbiota manifesting in an increase in the relative abundance of order *Clostridiales,* family *Ruminococcaceae* and *Lachnospiraceae* (see Figure 3-7). These bacteria have been previously shown to produce butyrate [207, 208]. Butyrate (one of the main SCFAs) may be protective from developing of obesity and metabolic disorder [181, 209]. Additionally, this probiotic in cheese was associated with a significant reduction on the family *Erysipelotrichaceae* and genus *Coprococcus*. The *Erysipelotrichaceae* abundance is high in obese individuals [210]. Furthermore, the relative abundance of *Coprococcus* is increased by high-fat diet [211]. These are

important considerations because they suggest the beneficial effect of the probiotic cow cheese supplementation on maintaining normal body weight gain and facilitating weight loss in obesity.

Overall, the results obtained and their bioinformatics analysis appear to support the conclusion that camel milk and cow cheese enriched with *Clostridium butyricum* probiotic are associated with potentially health beneficial changes of the mouse gut microbiota. The findings also suggest that the consumption of camel milk and the probiotic cow cheese may have beneficial effects on body weight gain, thus, providing the basis for future clinical trials to investigate their effects in prevention of obesity.

Author Contributions: TA conducted research, analyzed data and wrote the manuscript; EH designed the research, obtained funding, and contributed to data analysis and writing the manuscript; JGH conducted the gut microbiota analysis and contributed to data analysis and writing the manuscript; MR contributed to the dairy production and writing the manuscript; SMM contributed to manufacture of cheese and yogurt and editing the manuscript; INS contributed to data analysis, writing and revising the manuscript, and supervised TA for the research project. All authors provided critical feedback and helped shape the research, analysis, and read and approved the final manuscript.

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Conflict of Interest: The authors declare no conflict of interest.

CHAPTER 4. Effects of Food at Home vs. Food Away from Home-Induced Changes on Gut Microbiota and Immunological Status in Diabetes

Abstract: Gut microbiota dramatically affects our nutritional and immunological status. However, linking diet to the structure and dynamic operation of microbial communities and to human biology and pathobiology has proven elusive because of numerous uncontrolled genetic and environmental variables. To study whether the food at home (FAH), can help to reduce risk of developing diabetes by increasing abundance of the beneficial bacteria, T regulatory (T_{reg}) cells, and decreasing in inflammation-associated bacteria, IL-17 producing cells, and blood glucose levels, 12 healthy volunteers were randomly assigned into two diets, one based on food away from home (FAFH) and the other on food at home (FAH). Two weeks later, fecal samples of the volunteers were collected and analyzed, then transferred into antibiotic and non-antibiotic treated mice, and their resistance to develop diabetes was measured. The results obtained and their analysis support the conclusion that the FAH can help to reduce risk of developing diabetes by increasing abundance of the beneficial bacteria (*Bifidobacterium*), T_{reg} cells, and decreasing levels inflammation-associated bacteria (*Enterobacteriaceae*), IL-17 producing cells, and blood glucose levels. The antibiotics used in this study minimized result effects of FAH, emphasizing the link between gut microbiota, diet and immunity.

Keywords: food at home; food away from home; gut microbiota; diabetes; antibiotic; gut microbiome transplantation; T regulatory cells; IL-17; glucose tolerance test

4.1. Introduction

Gut microbiota dramatically affects the nutritional and immunological status of both humans and animals [212, 213]. Recent studies have shown that FAFH rich on fat and sugars are associated to dysbiosis (a microbial imbalance) and metabolic disease promoting [214-216], while microbiota and bacterial products founded on individuals with a diet high in fiber have anti-inflammatory properties [217-219]. Advances in "next generation" DNA sequencing have dramatically increased our capacity to study microbial communities associated with human body habitats (microbiota)[6, 7]. However, linking diet to the structure and dynamic operation of microbial communities and, more importantly, to the immunological status of the host has proven elusive because of numerous uncontrolled genetic and environmental variables and the intrinsically complicated nature of clinical trials. In the past, germ-free (GF) mice (animals born and raised without any exposure to micro-organisms) have provide an excellent system for controlling host genotype, diet and environmental conditions. In addition, they can be colonized at specific life stages with different microbial communities and thus perform comparative metagenomic studies of donor communities [123, 124]. However, raising and maintaining a GF mice colony is challenging. Hence, a broad range antibiotic treatment of mice is considered a viable alternative and has been successfully used elsewhere. Antibiotics can be administered by either oral or intraperitoneal route [125-127]. However, extrapolating results obtained in mice to humans is difficult because most bacterial genera and species found in mice are different than those found in humans [220]. Therefore, to develop an applicable mouse model, we propose to transplant human

fecal microbiota into antibiotic and non-antibiotic treated C57BL/6 mice. Once a stable "human-like" gut community is formed, these recipient animals will be evaluated for his resistance to develop metabolic disease, such as diabetes. We believe this unique model will allow us to unequivocally determine the effects of the diet on the microbial community and link it to the etiology of several metabolic disorders.

The objective of this study is to evaluate effects of food at home (FAH) and food away from home (FAFH) on human gut microbiota. In addition, to study whether the FAH-induced microbial and immunity changes can protect mice against diabetes. The results obtained and their analysis support the conclusion that the FAH can help to reduce risk of developing diabetes by increasing abundance of the beneficial microbial species, T regulatory cells, and decreasing IL-17 producing cell populations and blood glucose levels.

4.2. Materials and Methods

4.2.1. Human study participants and diets

Twelve healthy male, college student participants, were recruited for the study and assigned in a chronological order to the FAH group or FAFH group. FAH group (n=6) followed food prepared at home for the duration of the study (e.g., vegetables, fruits, nuts, seeds, legumes, brown rice, whole wheat breads, buttermilk, herbs, spices, fish, poultry, eggs, yogurt, seafood and extra virgin olive oil). Additionally, avoiding certain foods (e.g., soft drinks, added sugars, processed meat, refined grains, refined oils and other highly processed foods, fast food restaurants and frozen meals). The FAFH group (n=6) were the opposite of FAH and consumed at least 10 meals a week from national fast food chains, daily for two weeks **(Table 4-S1)**. All subjects gave their

informed consent for inclusion before they participated in the study. The study was approved by the SDSU IRB (Approval number: IRB-1512010).

Days	Restaurant Chains	Typical Meal	Calories
Wednesday 2/10/2016	McDonald's	Big Mac, large portion of French fries, large Coca Cola	1330 calories
Thursday 2/11/2016	Domino's	Italian Sausage & Pepper Trio Pizza, large Coca Cola	1410 calories
Friday 2/12/2016	Burger King	Triple Whopper Sandwich, large Coca Cola	1310 calories
Saturday 2/13/2016	McDonald's	Big Mac, large portion of French fries, large Coca Cola	1330 calories
Sunday 2/14/2016	Domino's	Italian Sausage & Pepper Trio Pizza, large Coca Cola	1410 calories
Monday 2/15/2016	Burger King	Triple Whopper Sandwich, large Coca Cola	1310 calories
Tuesday 2/16/2016	McDonald's	Big Mac, large portion of French fries, large Coca Cola	1330 calories
Wednesday 2/17/2016	Domino's	Italian Sausage & Pepper Trio Pizza, large Coca Cola	1410 calories
Thursday 2/18/2016	Burger King	Triple Whopper Sandwich, large Coca Cola	1310 calories
Friday 2/19/2016	McDonald's	Big Mac, large portion of French fries, large Coca Cola	1330 calories
Saturday 2/20/2016	Domino's	Italian Sausage & Pepper Trio Pizza, large Coca Cola	1410 calories
Sunday 2/21/2016	Burger King	Triple Whopper Sandwich, large Coca Cola	1310 calories
Monday 2/22/2016	McDonald's	Big Mac, large portion of French fries, large Coca Cola	1330 calories

Tuesday 2/23/2016	Domino's	Italian Sausage & Pepper Trio Pizza, large Coca Cola	1410 calories

Table 4-S1. Characteristics of FAFH group, including days of intervention, list of restaurant chains, typical meal, and calories.

4.2.2. Dietary assessments

At the study period, subjects were provided most of their food and instructed to record the contents of their daily intake for two weeks. Each subject's daily intakes of energy, macronutrients, and other nutrients were calculated from the food record and estimated by Food Processor Nutritional Analysis Pro version 11.4 (ESHA Research).

4.2.3. Animals, Antibiotic, fecal transplant, and diabetes inducing treatments

70-female 6-8-weeks old C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Upon arrival they were randomly grouped and keep in SPF filter cages and sterile bedding. Mice were fed standard low fat, plant polysaccharide-rich (LF/PP) diet *ad libitum,* and corresponding mice were treated with a broad range of antibiotic (Vancomycin 5mg/ml, Neomycin 10 mg/ml, Metronidazole 10 mg/ml, and Amphotericin-B 0.1 mg/ml) by oral gavage, daily for two weeks. In addition, water flasks were supplemented with 1g/L of ampicillin, to assure a consistent and efficient bacterial depletion [127]. Afterward, mice were colonized with fecal samples obtained from the human volunteers [221]. In order to reduce genetic variability, fecal samples within the same group were pooled, diluted in PBS (1g in 10 ml) and introduced by oral gavage (0.2 ml) into each recipient mice. Fecal samples were collected from the mice before and 24h after colonization and weekly after that. One week later diabetes were induced with streptozotocin (STZ), a compound that induces diabetes by inhibiting

insulin production on pancreatic β-cells [222] **(Table 4-1) (Figure 4-1).** Body weight was measured weekly, using top-loading balances with 0.01 g precision. The mice experiments were performed at the Animal Research Wing (ARW) facilities under the supervision of Michel Mucciante, DVM. The South Dakota State University Institutional Care and Use Committee approved the protocols and maintenance of the animals (IACUC Protocol number 15-094A).

Treatment	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Antibiotic cocktail	-	-	-	-	+	+	+	+
Fecal Transplant	-	FAH	FAFH	-	-	FAH	FAFH	-
Experimental condition	Diabetes	Diabetes	Diabetes	-	Diabetes	Diabetes	Diabetes	-
Number of mice	10	10	10	5	10	10	10	5

Table 4-1. Treatment groups and Experimental Design

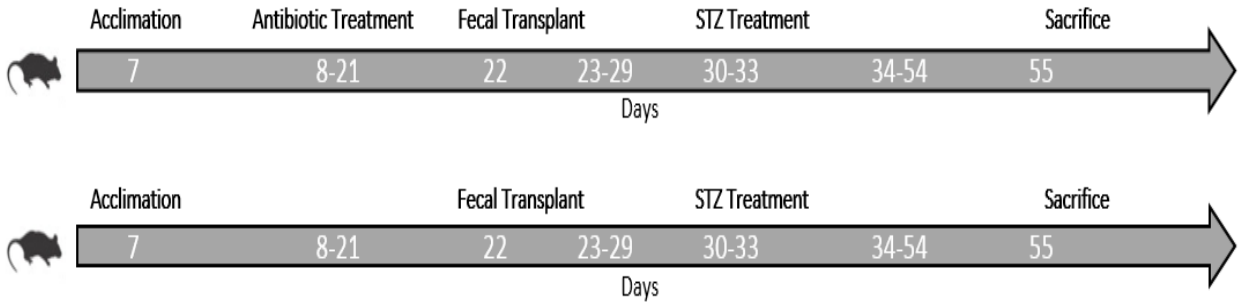

Figure 4-1. Time line of the experimental treatments

4.2.4. Flow cytometry and Cell preparation

Spleen tissues were collected from mice for the intracellular and external staining. The red cells was removed by ACK lysing buffer. The splenic cell suspension was stimulated and cultured for 5 h with leucocyte activation cocktail at 37 °C. Afterward, the cell was stained with FITC-CD4 and APC-CD25 antibodies (eBioscience, San Diego, CA) for 20 min at 4 °C. Then, cells were washed with FACS staining buffer (FB) (PBS containing 2% FCS and 0.02% NaN3) (BD PharMingen). For intracellular staining, cells were fixed and permeabilized using solution kit with BD GolgiStop™ (Cat. No. 554715), cells were incubated with rat anti-mouse IL-17A and rat anti-mouse Foxp3 antibody at 4 °C for 30-60 min, in dark condition. Then, the cells were washed and re-suspended in FB and acquired in a flow cytometer instrument. Data was analyzed using BD CSampler Software, (BD Biosciences, Mountain View, CA, USA).

4.2.5. Glucose Tolerance Test (GTT)

After 16-hours of fasting, GTT was performed. For the GTT, 10 mice were examined at each time point. Two g glucose per kg body weight was injected intraperitoneal. Blood glucose levels were obtained immediately before the injection (0 min) and at 15, 30, 60, and 120 min after glucose injection. The blood samples (5-µL) were collected via a small incision, made at the tip of the tail vein according to the manufacturer's instructions (FreeStyle glucose analyzer, Witney, Oxon, UK).

4.2.6. DNA Extraction and Real-Time PCR Analysis

A total of 24 human stool samples, twelve from each group (FAFH-FAH), were included in this study. DNA was extracted from stool sample by using microbial DNA extraction method (ZR Fecal DNA MiniPrep™) as previously described by [197]. All

primers and nucleotide sequences used for real-time PCR are shown in **(Table 4-2)**. PCR amplification was carried out by (Agilent Technologies M x 3005P qRT-PCR) in the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Power SYBR Green PCR Master Mix (AB # 4367659, 10µl for 1 reaction). Primer (16 µM/µl- 0.5 F + 0.5 R for 1 reaction) and 9µl of each cDNA sample. All data were analyzed by using the comparative C_T method [223]. As following:

$$\text{Fold change} = 2^{\Delta\Delta C_T}$$

$$\Delta\Delta C_T = [(C_T \text{ target} - C_T \text{ ref})_{\text{sample A}} - (C_T \text{ target} - C_T \text{ ref})_{\text{sample B}}]$$

Primer	Sequence 5'-3'
UniF	GTGSTGCAYGGYYGTCGTCA
UniR	ACGTCRTCCMCNCCTTCCTC
Ptgs2 For	TGGGTTCACCCGAGGACTGG
Ptgs2 Rev	TCGCACACTCTGTTGTGCTCC
Reg3b For	CCCTCCGCACGCATTAGTTG
Reg3b Rev	GCCTCAGCGCTATTGAGCAC
Il6 For	AAA GAG TTG TGC AAT GGC AAT TCT
Il6 Rev	AAG TGC ATC ATC GTT GTT CAT ACA
Tnfa For	AGTGGTGCCAGCCGATGGGTTGT
Tnfa Rev	GCTGAGTTGGTCCCCCTTCTCCAG
Cxcr3 For	CTCTTTGCCCTCCCAGATTTC
Cxcr3 Rev	GGCATAGCAGTAGGCCATGAC
2B4 For	ATGTTCAGCTCCCTTCTAG
2B4 Rev	GAGTTCCTCCCTGGCAGATC
Gp49b For	ACTCACAGCATCAGGCCAAT
Gp49b Rev	ACTGGTATCCGATGAGGATG
Bifidobacterium For	CGGGTGAGTAATGCGTGACC
Bifidobacterium Rev	TGATAGGACGCGACCCCA
Enterobacteriaceae For	GTGCCAGCMGCCGCGGTAA
Enterobacteriaceae Rev	GCCTCAAGGGCACAACCTCCAAG

Table 4-2. Primer sequences for real-time PCR

4.2.7. Statistical analysis

A one-way ANOVA with independent samples *t*-test was used for group comparison of the T regulatory cells, IL-17 producing cells, glucose tolerance test, and body weight. Unpaired *t*-test was used for the group comparison between FAFW and FAH for nutrients intake and gut microbiota. The results were expressed as means ± S.E, and mean differences were considered significant at $p < 0.05$. Data were analyzed using (SPSS Statistics, v. 25).

4.3. Results

In this study, we evaluated effects of FAH and FAFH diets on human gut microbiota. In addition, we study whether the FAH diet-induced microbial changes can protect mice against diabetes by modulation of the immune system. Twelve participants were recruited in the study (6 in the FAH group and 6 in the FAFH group). The average age and body mass index BMI of the study participants was 26.6 years and 26.9 (kg/m^2) respectively. No participants dropped out of the study during the two weeks intervention period.

4.3.1. Estimated Nutrients Intake at FAH and FAFH Groups of Intervention Periods

At the study period, subjects were provided most of their food and instructed to record the contents of their daily intake for two weeks. Evaluation of dietary intakes of the study subjects through the study revealed no significant variations in calories, carbohydrates, and cholesterol intakes between the two groups **(Table 4-3)**. As expected, due to a consumption of food prepared at home for the duration of the study, there was a significant increased on total dietary fiber intakes compared to FAFH group ($p < 0.001$).

In addition, there were a significant increased on the fat ($p < 0.05$), saturated fat ($p < 0.004$), and trans fatty acid ($p < 0.03$) intakes in FAFH group compared to FAH. Also, a significant reduction in the intakes of monounsaturated fat ($p < 0.05$) and polyunsaturated fat ($p < 0.03$) were observed in FAFH group compared to FAH group **(Table 4-3)**. These results indicate that the FAH diet used in the study altered nutrients of intake, some of which can be associated with health benefits (particularly, significantly increased nutrients of dietary fiber and mono/polyunsaturated fat due to the FAH diet intervention).

Nutrients	**FAH**	**FAFH**	***p* * : FAH *vs* FAFH**
Calories (kcal/d)	2107 ± 97	2164 ± 50	NS
Protein (g/d)	105 ± 12	72 ± 6	<0.05
Carbohydrates (g/d)	269 ± 45	257 ± 14	NS
Total Dietary Fiber (g/d)	29 ± 2	10 ± 1	<0.001
Fat (g/d)	73 ± 10	100 ± 5	<0.05
Saturated Fat (g/d)	20 ± 4	42 ± 4	<0.004
Monounsaturated Fat (g/d)	17 ± 4	5 ± 4	<0.05
Polyunsaturated Fat (g/d)	9 ± 1	2 ± 2	<0.03
Trans Fatty Acid (g/d)	0.008 ± 0.001	1 ± 0.41	<0.03
Cholesterol (mg/d)	328 ± 88	253 ± 29	NS

Table 4-3. Estimated nutrients intake at FAH and FAFH groups of intervention periods. FAH, food at home; FAFH, food away from home; NS, not significant. The results are expressed as mean ± SE. *p** Obtained from independent *t*-test (SPSS Statistics, v. 25).

4.3.2. Effect of FAFH and FAH diets on human gut microbiota

Gut microbiota analysis was conducted using quantitative PCR with specific primers for *Bifidobacterium* and *Enterobacteriaceae*. The gene expression of *Bifidobacterium* was noticeably enriched after FAH intervention **(Figure 4-2A)**, while the *Enterobacteriaceae* showed a decreasing trend after FAH intervention **(Figure 4-2B)**. Our study indicates that short-term (2 weeks) FAH intervention results in modulation of the gene expression, accompanying by an increase in beneficial bacteria (*Bifidobacterium*) and a decrease in inflammation-associated bacteria (*Enterobacteriaceae*).

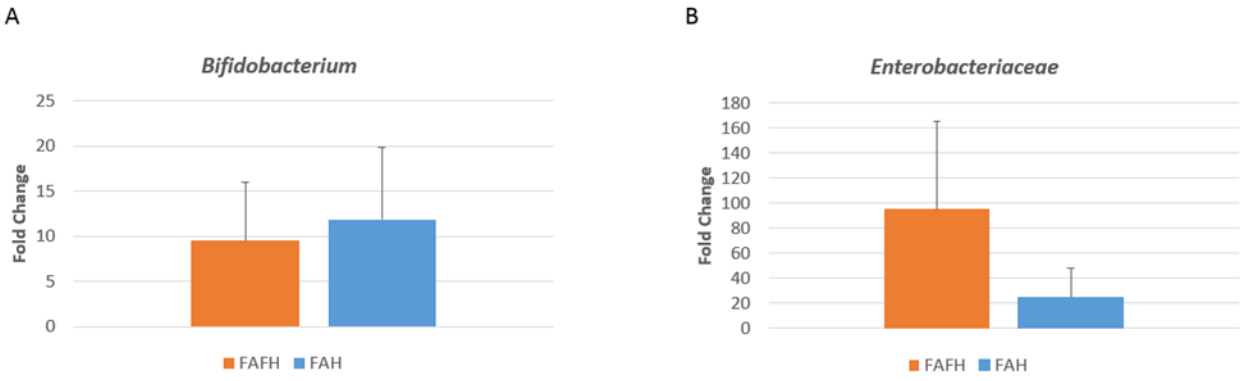

Figure 4-2. Mean fold change of gene expression was calculated using the comparative C_T method (2ΔΔCt) between the intervention group, for *Bifidobacterium* (A), and *Enterobacteriaceae* (B). The results are expressed as mean ± SE. Unpaired t-test was used for the group comparison (SPSS Statistics, v. 25).

4.3.3. Effect of FAFH and FAH diets on Gp49b, 2B4, Reg3b, Ptgs2, Cxcr3, Tnfa, and Il6 parameters

Parameter analysis was conducted using quantitative PCR with specific primers for *Gp49b, 2B4, Reg3b, Ptgs2, Cxcr3, Tnfa,* and *Il6*. The results attempt to confirm the effect of FAFH and FAH diets on this parameters, such a links of this parameters have been incorporated in health and diseases. The quantity of *Gp49b, 2B4, Reg3b,* and *Ptgs2* were significantly higher in the FAFH than that in FAH subjects **(Figure 4-3. A, B, C,** and **D)** (p=0.01) (p=0.03) (p=0.05) (p=0.04) respectively. While the level of *Cxcr3* and *Tnfa* were significantly higher in the FAH group compared to the FAFH **(Figure 4-3. E** and **F)** (p=0.05) (p=0.01) respectively. No significant difference was perceived on fold change of *Il6* between the FAFH and FAH subjects, even though this fold change was relatively abundant in the FAFH group compared to the FAH group **(Figure 4-3G)**. These results indicate that the FAH diet used in the study altered gene expression of parameters, some of which can be associated with health benefits (particularly, significantly decreased parameters of Glycoprotein (*Gp49b*), Regenerating Islet-Derived 3 Beta (*Reg3b*), and Prostaglandin-Endoperoxide Synthase 2 (*Ptgs2*) due to the FAH diet intervention).

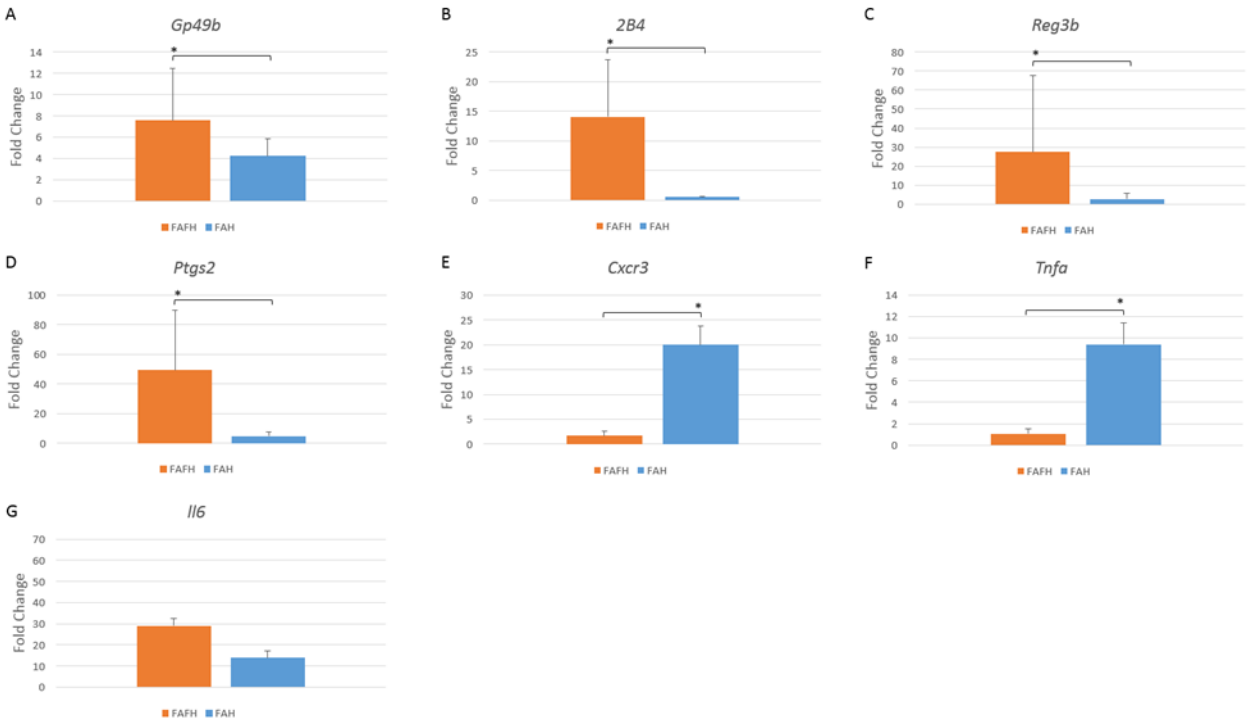

Figure 4-3. Mean fold change of gene expression was calculated using the comparative C_T method (2ΔΔCt) between the intervention group, for *Gp49b* (A), *2B4* (B), *Reg3b* (C), *Ptgs2* (D), *Cxcr3* (E), *Tnfa* (F), and *Il6* (G). The results are expressed as mean ± SE. $^{*}p < 0.05$. Unpaired t-test was used for the group comparison (SPSS Statistics, v. 25).

4.3.4. Effect of $CD4^{+}CD25^{+}Foxp3^{+}$ T regulatory cells

We here studied and compared whether production of $CD4^{+}CD25^{+}Foxp3^{+}$ T regulatory cells can be influenced by antibiotic or non-antibiotic treatment, diabetes, FAT and FAFH diet. T regulatory cells are a subpopulation of T cells which have a role in suppressing or controlling other cells, to prevent autoimmune disease [224, 225]. The results obtained indicate that there were no statistically significant differences between the groups of (Control, Antibiotic + Fecal Transplant FAH + Diabetes, and Antibiotic + Fecal Transplant FAFH + Diabetes) **(Figure 4-4A).** On the other hand**,** the percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ T regulatory cells was significantly increased in Fecal Transplant

FAH + diabetes group compared to Fecal Transplant FAFH + diabetes (p=0.009). Also, from Control compared to Fecal Transplant FAH + diabetes and Fecal Transplant FAFH + diabetes group (p=0.01) (p=0.001) respectively **(Figure 4-4B)**. The findings obtained demonstrate that FAH diet is effective for increasing the levels of $CD4^{+}CD25^{+}Foxp3^{+}$ T regulatory cells, but not in mice treated with antibiotic.

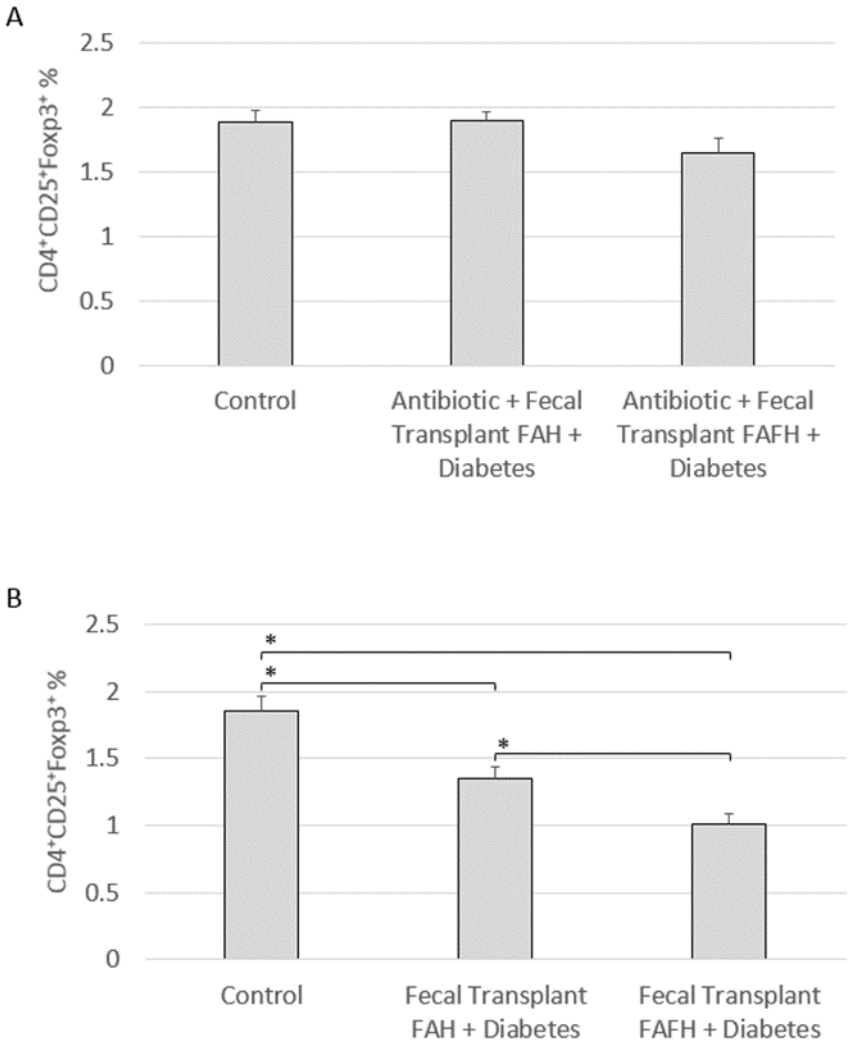

Figure 4-4. Percentage of $Foxp3^{+}$ cells within $CD4^{+}CD25^{+}$ T cells in each Control, Fecal Transplant FAH + Diabetes, and Fecal Transplant FAFH + Diabetes groups (n = 10) is shown. For antibiotic (A) and non-antibiotic treatment groups (B). The results are expressed as mean ± SE. A one-way ANOVA with independent *t*-test was used for the group comparison (SPSS Statistics, v. 25).

4.3.5. IL-17 production

We then studied and compared whether production of IL-17 cells can be influenced by antibiotic or non-antibiotic treatment, diabetes, FAH and FAFH diet. "IL-17 cells are $CD4^+$ T-helper cells that produce IL-17 family cytokines and other inflammatory cytokines. IL-17 producing cells are implicated in chronic inflammation and are considered to drive some autoimmune diseases"[226]. The results obtained indicate that there were no statistically significant differences between the groups of (Control, Antibiotic + Fecal Transplant FAH + Diabetes, and Antibiotic + Fecal Transplant FAFH + Diabetes) **(Figure 4-5A).** On the other hand**,** the percentage of IL-17 cells was significantly decreased in Fecal Transplant FAH + Diabetes group compared to Control, while Fecal Transplant FAH + diabetes group pointed to a decreasing trend in IL-17 cells percentage compared to Fecal Transplant FAFH + Diabetes group **(Figure 4-5B)**. The findings obtained demonstrate that FAH diet is effective for decreasing production of IL-17 cells, but not with mice treated with antibiotic.

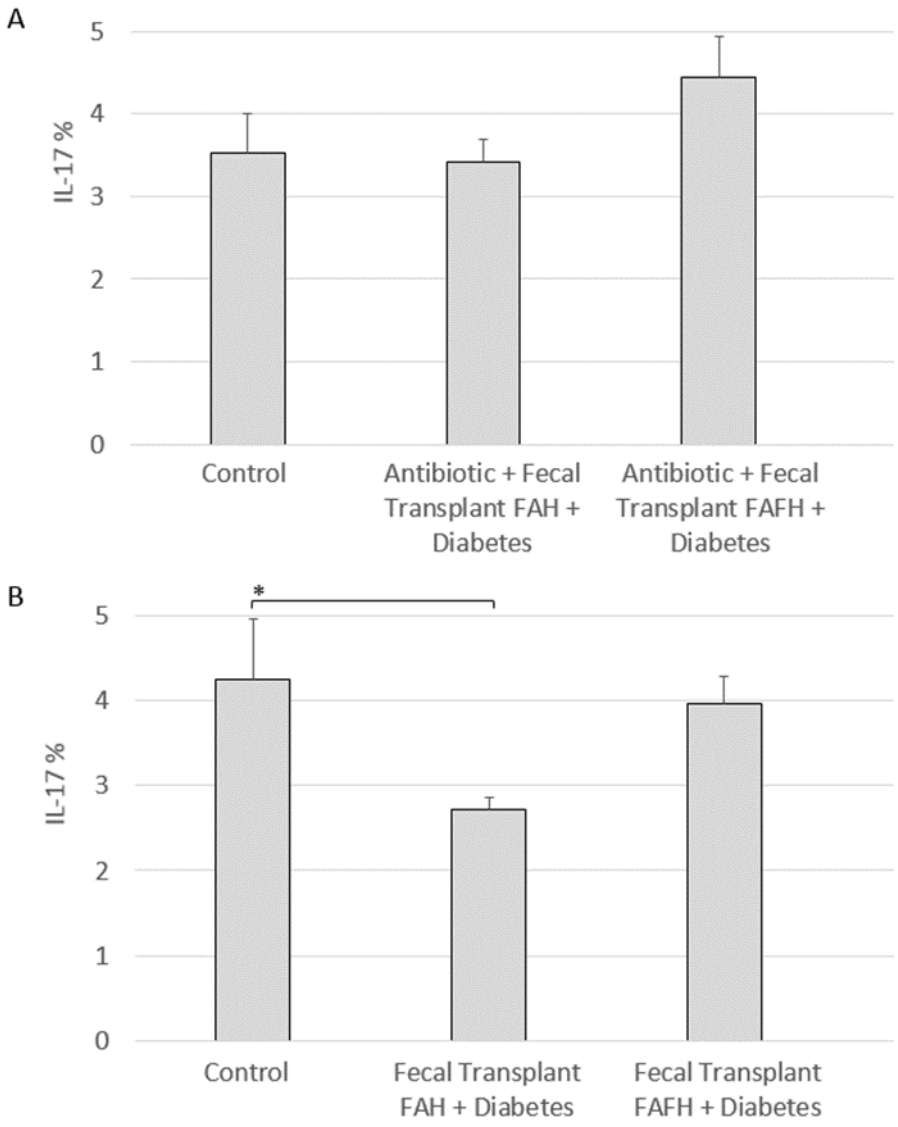

Figure 4-5. Percentage of IL-17 cells in each Control, Fecal Transplant FAH + Diabetes, and Fecal Transplant FAFH + Diabetes groups (n = 10) is shown. For antibiotic (A) and non-antibiotic treatment groups (B). The results are expressed as mean ± SE. A one-way ANOVA with independent t-test was used for the group comparison (SPSS Statistics, v. 25).

4.3.6. Glucose Tolerance Test (GTT)

Blood glucose levels were obtained immediately before the injection (0 min) and at 15, 30, 60, and 120 min after glucose injection. After the intraperitoneal injection of glucose, blood glucose levels were increased in each treatment group. At 15 min, the blood glucose level was significantly lower in the Fecal Transplant FAH + diabetes group than in the Fecal Transplant FAFH + diabetes group (p=0.01). In addition, it was also significantly lower in the Fecal Transplant FAH + diabetes group than in Control and Fecal Transplant FAFH + diabetes groups at 30 min (p=0.03) (p=0.02) respectively **(Figure 4-6A)**. On the other hand, the blood glucose levels of the group treated with antibiotic did not significantly differ between the groups **(Figure 4-6B)**. The findings obtained demonstrate that FAH diet is effective for decreasing blood glucose levels, but not with mice treated with antibiotic.

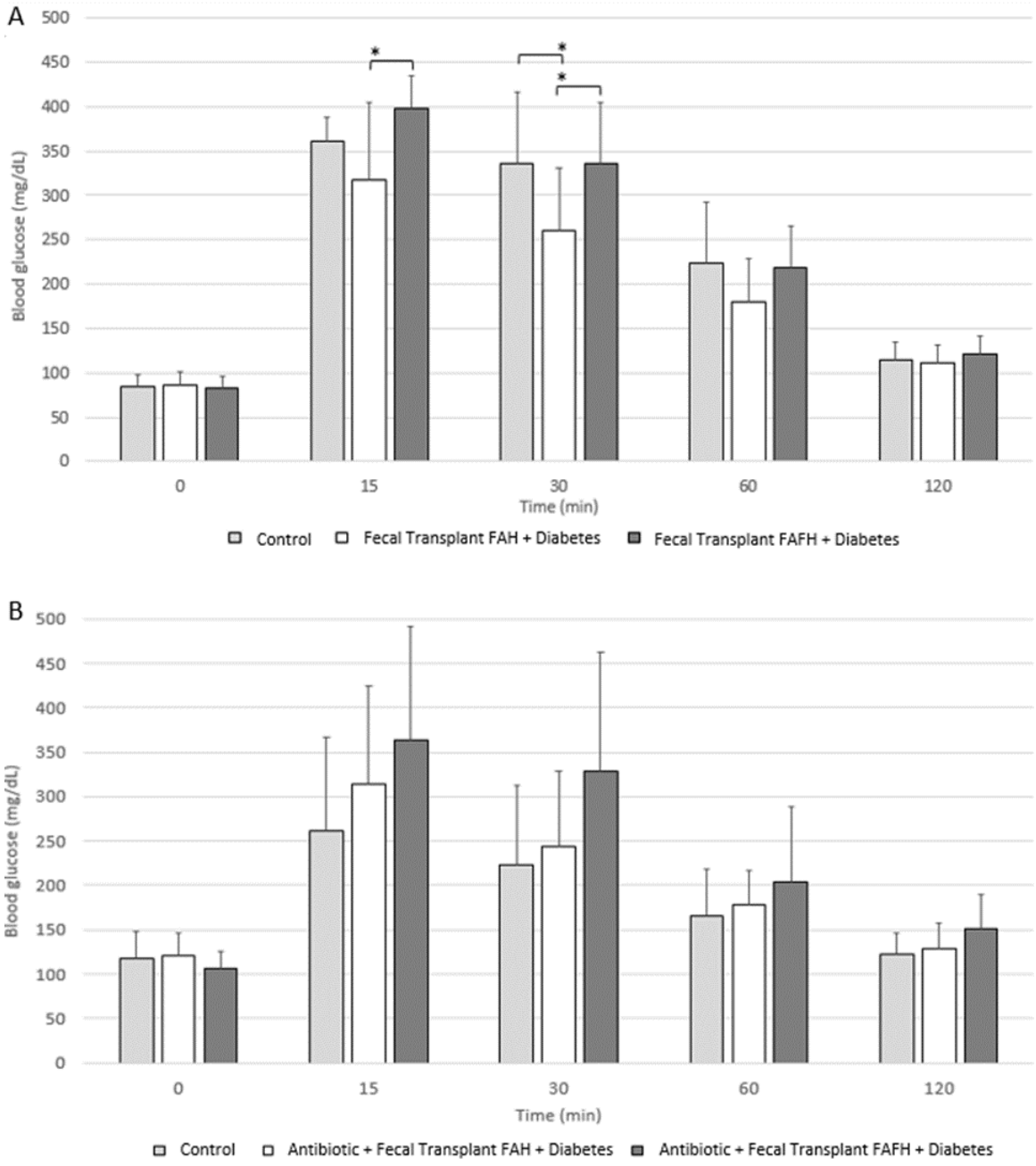

Figure 4-6. Glucose tolerance test in each Control, Fecal Transplant FAH + Diabetes, and Fecal Transplant FAFH + Diabetes groups (n = 10) is shown. For non-antibiotic (A) and antibiotic treatment groups (B). The results are expressed as mean ± SE. A one-way

ANOVA with independent *t*-test was used for the group comparison (SPSS Statistics, v. 25). **4.3.7. Body Weight and Days of Intervention**

Body weight was measured weekly, using top-loading balances with 0.01 g precision. As seen in **figure 4-7**, there were no significant difference on the body weight between the groups treated and not treated with antibiotic (**Figure 4-7A**) (**Figure 4-7B**). However, the body weight gain of the Fecal Transplant FAH + Diabetes group exhibited a decreasing trend at day 22.

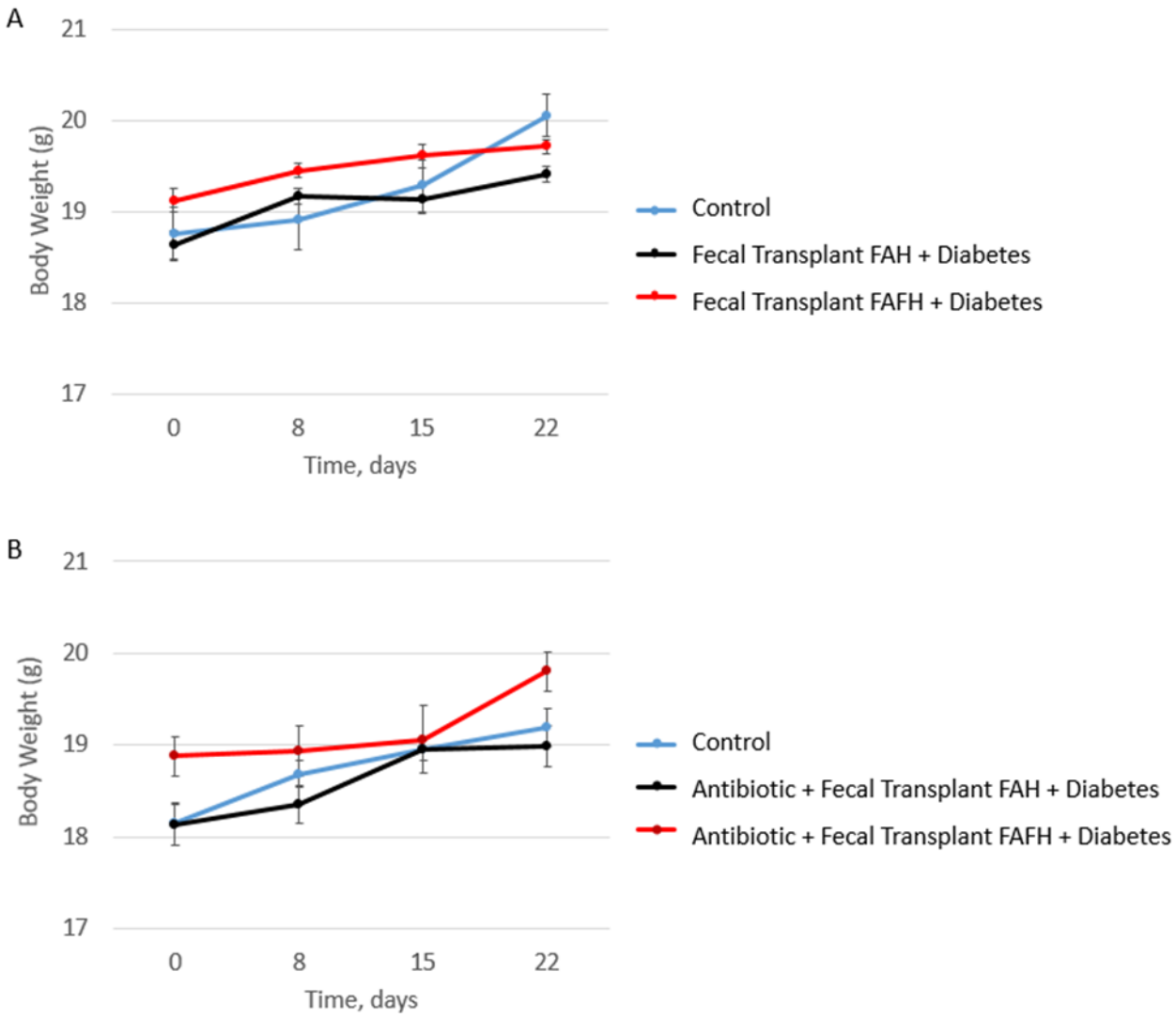

Figure 4-7. Body weight in each Control, Fecal Transplant FAH + Diabetes, and Fecal Transplant FAFH + Diabetes groups (n = 10) is shown. For non-antibiotic (A) and antibiotic treatment groups (B). The results are expressed as mean ± SE. A one-way ANOVA with independent t-test was used for the group comparison (SPSS Statistics, v. 25).

4.4. Discussion

The study was designed to evaluate the effects of food at home (FAH) and food away from home (FAFH) on human gut microbiota. In addition, to study whether the FAH diet-induced microbial and immunity changes can protect mice against diabetes following the transplantation of human fecal microbiota into antibiotic and non-antibiotic treatment mice. While FAH diet have been shown to improve markers of metabolic syndrome and gut microbiota [227, 228]. However, this study was comprehensively evaluating effects of FAH and FAFH diets in gut microbiota, immune function, body weight, diabetes developing in antibiotic and non-antibiotic treatment mice.

An important finding of the study is that the FAH group resulted in a noticeable increase in abundance of *Bifidobacterium* strains in the gut after a 2-weeks intervention. It has been stated that some strains of *Bifidobacterium* can be referred as healthy gut microbiota along with anti-diabetes agents [229, 230]. Furthermore, *Enterobacteriaceae* family was noticeably decreased after the FAH diet intervention (see Figure 2-2). Previous studies have stated that these family are associated with inflammation-driven bacterial dysbiosis and positively correlated with diabetes [231-233]. Therefore, the study confirmed that short-term (2 weeks) FAH intervention results in an increase in beneficial bacteria (*Bifidobacterium*) and a decrease in pro-inflammatory bacteria (*Enterobacteriaceae*).

Dietary patterns containing FAH diet are now broadly recognized to be critical for protection against various diseases with an inflammatory nature. In contrast, FAFH diet are recognized to apply various harmful effects [234, 235]; including its metabolic and inflammatory related alterations in $CD4^{+}CD25^{+}Foxp3^{+}$ T regulatory cells, and IL-17

[236]. Therefore, we examined the possible alterations caused by FAH diet to this populations and there resistance for development of diabetes. Higher percentage of $CD4^+CD25^+Foxp3^+$ T regulatory cells were observed in Fecal Transplant FAH + diabetes group compared to Fecal Transplant FAFH + diabetes (see Figure 4-4B). In the Fecal Transplant FAH + diabetes group, increasing percentage of $CD4^+CD25^+Foxp3^+$ T regulatory cells was associated with decreasing blood glucose levels (see Figure 4-6A). However (and somewhat paradoxically), higher percentage of $CD4^+CD25^+Foxp3^+$ T regulatory cells at control group compared to Fecal Transplant FAH + diabetes and Fecal Transplant FAFH + diabetes were also observed (see Figure 4-4B). The administration of diabetes (STZ) and the effect of longstanding hyperglycemia can promote a strong inflammatory progression in the islets which is toxic to beta cells. Thus, increasing the number of T regulatory cells in the spleen were as a results of controlling damage of the tissue [237]. The role of IL-17 producing cell in FAH diet has not been totally examined. Thus, our data point out an increased on the frequencies of IL-17 cell in control group compared to Fecal Transplant FAH + diabetes; which also involve in the pathogenesis of diabetes.

In the present study, the FAH diet used in the study altered gene expression of parameters, some of which can be associated with health benefits (particularly, significantly decreased parameters of *Gp49b, Reg3b,* and *Ptgs2* due to the FAH diet intervention)[238-240]. An interesting finding was the reduced glucose intolerance in FAH fecal transplant, *Gp49b, Reg3b,* and *Ptgs2* –deficient mice. Therefore, *Gp49b, Reg3b,* and *Ptgs2* might be important for the development of type 2 diabetes which requires further investigation.

Our data point out that antibiotic administration minimized result effects of FAH diet on both $CD4^{+}CD25^{+}Foxp3^{+}$ T regulatory cells, IL-17 production, and glucose tolerance test, compared to those groups of non-treated with antibiotic, such an antibiotics can change the population or balance of composition and more compromise mucosal immunity of the gut microbiota [241].

Evaluation of dietary intakes of the study subjects through the study revealed a significant variations between the two groups (Table 4-3). As expected, due to a consumption of food prepared at home FAH, there was a significant increased on total dietary fiber intakes compared to FAFH group. These results indicate that the FAH diet used in the study altered nutrients of intake, some of which can be associated with health benefits (particularly, significantly increased nutrients of dietary fiber due to the FAH diet intervention). Importantly, these dietary fiber have been previously shown to produce short-chain fatty acids (SCFAs), a bacterial fermentation product of dietary fiber, recognized to have wide-ranging effects on maintenance of health and host physiology [199-201].

The design of the study has allowed us to detect important effects of FAH vs. FAFH-induced changes on gut microbiota and immunological status in diabetes in the relatively limited number of participants in this study. However, the used of dietary records are affected by error and has limitation “due mainly to the tendency of subjects to report food consumption close to those socially desirable. Further problems are related to the high burden posed on respondents. It can also influence food behavior in respondents in order to simplify the registration of food intake and some subjects can experience

difficulties in writing down the foods and beverages consumed or in describing the portion sizes" [242].

The results obtained and their analysis support the conclusion that the FAH can help to reduce risk of developing diabetes by increasing abundance of the beneficial bacteria (*Bifidobacterium*), T regulatory cells, and decreasing in inflammation-associated bacteria (*Enterobacteriaceae*), IL-17 producing cell populations, and blood glucose levels. The antibiotics used in this study minimized result effects of FAH diet. Further additional studies using germ-free mice are needed to better understand the role of gut microbiota in the modulation of the immune system and its contribution to inflammation and autoimmunity diseases.

CHAPTER 5. General Discussion

The rising prevalence of obesity and metabolic disorders and its harmful health consequences are of increasing global concern [1, 2]. The health effects of being overweight and obese are not without controversy [243, 244], but large pooling studies have shown increased risks for cardiovascular diseases, diabetes, cancers and chronic kidney diseases[245, 246]. Although public health campaigns and an increasingly health-conscious general population has succeeded in slowing the growth rate of obesity in developed countries, much more has to be done in order to reach the WHO target of halting the rise in obesity by 2025. To date, there is not a single country with well-documented downwards trends in obesity rates. Therefore, rigorous data-based plans combining a healthy diet with the promotion of a more active lifestyle are needed in order to be evaluated and translated into national obesity control programs.

From a metabolic point of view, obesity is the consequence of a prolonged imbalance between energy intake and expenditure caused by a very complex interplay between genetics, nutrition and environmental factors [247, 248]. Recent studies have shown that gut microbiota plays a critical role in the pathogenesis of obesity and associated diseases [249]. Such a link was first suggested after observing that colonization of lean mice with cecal microbiota from obese mice resulted in a greater increase in body fat when compared with microbiota from lean mice [18]. Subsequent studies in both mice and humans confirmed that obesity was accompanied by an altered gut microbiota [153, 250-252]. Therefore, the gut microbiota has become a target for obesity and metabolic disease prevention. Indeed, data from both experimental and clinical studies suggest that modulation of gut microbiota through administration of

probiotics (normally *Lactobacillus* or *Bifidobacterium*) may be an effective strategy to treat metabolic diseases, although other studies failed to find a correlation between probiotic supplements and an improved health status [253]. Besides probiotics, the gut microbiota can be modulated through administration of prebiotics, food ingredients that resist degradation in the upper digestive tract and selectively enhance the growth and/or activity of one or few resident bacteria and can improve insulin sensitivity, lipid metabolism and low-grade chronic inflammation [254, 255]. Consequently, synbiotics, or the combination of both pre and probiotics, have the potential to induce a more substantial effect on gut microbiota and host health and are therefore the focus of a big research effort [256].

Probiotic bacteria have often been assessed as dietary supplements or in dairy products, such as yogurt or kefir [115, 116]. We believe that because of its nutritional values and longer shelf life, fermented cheese would be an ideal vector for the delivery of probiotics. Cheese is a good matrix for transferring probiotic bacteria into the gastrointestinal tract, due to the buffering capacity of the milk proteins which will protect the cells during transit; this is mainly due to their higher fat content and denser structure that may protect the bacteria against the acidic environment of the gastrointestinal tract [117-119].

It was demonstrated that whole grain diet can decreased risk of numerous lifestyle-associated diseases including type 2 diabetes, for the reason that “microbial degradation of whole grains, rich in dietary fibers, leads to production of short-chain fatty acids, which may exert beneficial effects on the host metabolism” [257]. However, it is not been thoroughly investigated to what extent beneficial effects of whole grain and

food at home consumption are associated with the gut microbiota and immunological status.

In view of the potential effects of diet, prebiotics, and probiotics on gut microbiota in obesity and metabolic disorders, the main hypothesis tested in these studies was that the whole grain diet, dairy products, pre and probiotics will modulate gut microbiota by increasing abundance of the beneficial microbial species and the supplement may also have beneficial effects on body composition, immune system, and metabolic parameters in obesity. The **aims** of this dissertation were (1) to evaluate effects of the synbiotic supplement containing *Bifidobacterium* and *Lactobacillus* strains on the human-gut microbiota in relation to changes in body composition and metabolic biomarkers in obesity, (2) to characterize the effect of dairy products (cow, goat, and camel milk and fermented cheese and yogurt originated from cow milk and containing the well-established probiotic *Clostridium butyricum*) on taxonomic composition and relative abundance of the mouse gut microbiota and body weight, (3) to study the effects of food at home and food away from home diets on human gut microbiota. In addition, to study whether the food at home-diet-induced microbial and immunity changes can protect mice against diabetes.

The data presented in **Chapter 2** indicate that weight loss in human subjects participating in a high-protein, low-carbohydrate, energy-restricted eating weight loss program is accompanied by changes in gut microbiota that can be associated with increased genotoxicity [144]. The synbiotic used in this study modulated the human gut microbiota by increasing abundance of the microbial species that can be considered to be of benefit to their host; it was also associated with positive effects on metabolic

parameters in obesity. A combination of the four strains of *Bifidobacterium* and *Lactobacillus acidophilus* in the synbiotic supplement resulted in a significant increase in abundance of these probiotic genera in the gut after a 3-month intervention. *Bifidobacterium* is largely considered a positive member of the microbial community and furthermore, there has been some association with anti-obesity effects [162, 166, 167]. In addition to this, further modulation of the microbiota was observed, for example, *Prevotella* and *Gardnerella* genera were significantly decreased after the synbiotic intervention. Previous studies have reported that these genera are associated with chronic inflammatory conditions and positively correlated with obesity [168-170]. Therefore, the reduction in these genera could help to modulate the balance to improve metabolism within the host. Special caution is warranted when analyzing the data referring to *Prevotella*, a complex genus linked both to health and disease and maybe influenced by race/ethnicity [171]. However, statistically significant differences in the community composition of gut microbiota between groups (synbiotic vs. placebo) and time points (end vs. beginning of trial) using parameters of alpha-diversity and beta-diversity were not observed. Our data is compatible with a recent study that did not found a relationship between severe caloric restriction and changes in alpha-diversity[172]. In addition, correlation and regression analyses did not indicate statistically significant or apparently beneficial associations between species contained in the synbiotic supplement (*Bifidobacterium* and *Lactobacillus*) and body composition parameters, including at the end of synbiotic intervention. Interestingly, the changes over time in body mass, BMI, waist circumstance, and body fat mass demonstrated a positive correlation trend with *Bifidobacterium* abundance in the synbiotic group, while changes in body fat mass were

negatively correlated with *Lactobacillus* abundance in the placebo group. However, positive associations between relative abundance of *Bifidobacterium* and several body composition parameters appear to point to the unfavorable role of these bacteria in promoting weight loss, although any potential benefits of this genera could be masked by the high-protein diet used in the study. High protein intake induces proteolytic fermentation in the gut with synthesis of compounds that have been implicated in the development of obesity and metabolic syndrome and modulating the gut microbiota [140-142, 173] and the production of toxic metabolites [174]. Several studies have also found that an increase in *Bifidobacterium* and *Lactobacillus* abundance is correlated with both pro- and anti-obesity effects in obese human subjects [175, 176], thus complicating the interpretation of the results. Individual differences in energy extraction may contribute to explain the observed differences [177]. Additionally, Bifidobacterium have been linked with improved barrier function in overweight individuals, thus adding a potential beneficial mechanism of action [81]. Therefore, more studies are needed to fully understand the observed divergences and correctly identify human subpopulations susceptible to benefit from synbiotic intervention.

Regression analysis performed to correlate microbial abundance of species contained in the synbiotic supplement with biomarkers of obesity found a novel significant association between a decrease over time in HbA1C percentage and an increase in Lactobacillus abundance, particularly in the synbiotic group. This is an important observation because it demonstrates the beneficial effect of increasing *Lactobacillus* abundance on potentially reducing blood glucose levels. Negative associations between *Megasphaera* abundance and *Eubacterium ruminantium* abundance

with HbA1C levels were observed in the synbiotic group at the end of trial. *Eubacterium ruminantium* are xylanolytic bacteria (i.e., producing xylanase following dietary fiber fermentation) and Megasphaera bacteria utilize lactate [178], which can underlie potential relationship of these species to decreasing blood glucose levels [179, 180]. However, within the trial following the synbiotic a decrease in *Megasphera* was observed, an increase in *Eubacterium ruminantium*. This could imply that the synbiotic intervention and associated microbial changes could be linked to maintaining a normal blood glucose levels in obesity.

It should also be considered that the microbial shifts observed in this study may be associated with a positive impact on microbial fermentation within the large intestine. Whilst the microbial changes observed following synbiotic intervention included an increase in *Ruminococcus*, a genera known to produce butyrate. Butyrate is a short chain fatty acid that provides and energy source for the colonocytes, as a histone deacetylase inhibitor this SCFA is linked to anti-cancer effects, hence could offer protection against some of the metabolites that are produced within a high protein diet. SCFA's have also recently been associated with protection against type 1 diabetes [181]. In addition to this lactobacilli and bifidobacteria are associated with positive effects of colonic health and following synbiotic intervention have been associated with reducing fecal water genotoxicty, which is considered a biomarker for colon cancer [182]. Therefore, whilst within the weight-loss diet employed the synbiotic treatment may have had a limited impact on the weight-loss parameters, it is possible that the changes in the microbiota could help to reduce detriments associated with a high protein diet.

Manipulation of the gut microbiota through dietary intervention and probiotic supplements can be a promising strategy for the prevention and treatment of metabolic syndrome and obesity. Therefore, data presented in **Chapter 3** demonstrated that gut microbiota enrichment can be achieved via consumption of camel milk, as compared with cow and goat milk. In addition, the significant gut microbiota enrichment was observed in mice supplemented with the probiotic cow cheese, as compared with the probiotic cow and goat yogurt. To our knowledge, this is the first study that compared the effects of supplementation with dairy products originating from different animals on the gut microbiota composition, particularly, the study of camel milk and fermented cheese. The present study indicated that, when compared with mice fed with cow milk, mice fed a diet supplemented with camel milk demonstrate a decrease in body weight gain accompanied by characteristic changes in gut microbiota, including an increase in order *Clostridiales* and genus *Anaerostipes*. Importantly, these bacteria have been previously shown to produce short-chain fatty acids (SCFAs) a bacterial fermentation product of dietary fiber, recognized to have wide-ranging effects on maintenance of health and host physiology [199-201]. We also found a decreased abundance of *Peptostreptococcaceae* bacteria in the camel milk group. Importantly, species in this family are positively associated with obesity and inflammation [202-204]. The health benefits of camel milk may be partly underlined by its functioning as prebiotic. Camel milk has a high overall nutritional value and high heat stability in the process of preservation of raw milk by pasteurization [205, 206]. The induction of the SCFAs producing gut bacteria via consumption of camel milk may lead to development of nutritional strategy for weight loss and the prevention and treatment of obesity.

Surviving passage over the host's gastrointestinal tract is a critical event for any probiotic. In this regard, we observed that the total significant richness in the dataset were 16 OTUs for the probiotic cow yogurt group vs. the cow yogurt, 13 OTUs for the probiotic goat yogurt group vs. the goat yogurt group, and 30 OTUs for the probiotic cow cheese group vs. the cow cheese group. These results indicate that the probiotic supplement used in the study modified relative abundance of gut bacteria, some of which can be associated with health benefits (particularly, significantly increased abundance of order *Clostridiales* due to the probiotic intervention). Therefore, cheese appear to be a good matrix for transferring probiotic bacteria into the gastrointestinal tract, probably, due to the high buffering capacity of milk proteins which will protect the microbial cells during transit (high fat cheese content and dense product structure may also protect the bacteria against the acidic environment in the gastrointestinal tract [117-119]). *Clostridium butyricum* supplementation triggered a shift in body weight gain with a significant decrease in probiotic cow cheese group compared with the probiotic goat and cow yogurt groups. We also found that the addition of this probiotic to cow cheese improves gut microbiota manifesting in an increase in the relative abundance of order *Clostridiales*, family *Ruminococcaceae*, and *Lachnospiraceae*. These bacteria have been previously shown to produce butyrate [207, 208]. Butyrate (one of the main SCFAs) may be protective from developing of obesity and metabolic disorder [181, 209]. Additionally, this probiotic in cheese was associated with a significant reduction on the family *Erysipelotrichaceae* and genus *Coprococcus*. The *Erysipelotrichaceae* abundance is high in obese individuals [210]. Furthermore, the relative abundance of *Coprococcus* is increased by high-fat diet [211]. These are important considerations because they suggest

the beneficial effect of the probiotic cow cheese supplementation on maintaining normal body weight gain and facilitating weight loss in obesity.

The data presented in **Chapter 4** indicate that the food at home, can help to reduce risk of developing diabetes by increasing abundance of the beneficial bacteria (*Bifidobacterium*), T regulatory cells, and decreasing in inflammation-associated bacteria (*Enterobacteriaceae*), IL-17 producing cells, and blood glucose levels. Importantly, the FAH diet used in the study altered gene expression of novel parameters, some of which can be associated with health benefits (particularly, significantly decreased parameters of *Gp49b, Reg3b,* and *Ptgs2* due to the FAH diet intervention)[238-240]. An interesting finding was the reduced glucose intolerance in FAH fecal transplant, *Gp49b, Reg3b,* and *Ptgs2* –deficient mice. Consequently, *Gp49b, Reg3b,* and *Ptgs2* might be important for the development of type 2 diabetes which requires further investigation. Our data also point out that antibiotic administration minimized result effects of FAH diet on both $CD4^+CD25^+Foxp3^+$ T regulatory cells, IL-17 producing cells, and glucose tolerance test, compared to those groups of non-treated with antibiotic, such an antibiotics can change the population or balance of composition and more compromise mucosal immunity of the gut microbiota [241]. Further additional studies using germ-free mice are needed to better understand the role of gut microbiota in the modulation of the immune system and its contribution to inflammation and autoimmunity diseases.

In summary**,** this dissertation explores a scope of studies on the effects of diet on gut microbiota and health outcomes including obesity and metabolic disorders taking into perspective diets such as food at home, dairy products, high-protein, low-carbohydrate, prebiotic and probiotic. The main outcome of our studies is identification of an effective

and novel approach for the prevention and treatment of obesity and metabolic disorders that is based on modulating the human/mouse gut microbiota and increasing abundance of the microbial species that can be considered to be of benefit to their immune system and host.

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APPENDIX

Table 2-S3. Body composition parameters, including DXA scans data. A and B before sample numbers indicate the synbiotic and placebo groups, respectively.

Treatment	Timepoint	Subject	Gender	Age	HbA1C_percent	Body_mass_kg	BMI_kg_per_m2	WC_cm	Body_Fat_percent
Synbiotic	EndOfTrial	A1	F	48	4.5	63.8	26.4	97	33.9
Synbiotic	EndOfTrial	A10	F	66	5.5	70.3	28.4	93	41.6
Synbiotic	EndOfTrial	A11	M	45	4.9	82.5	26.9	102	NA
Synbiotic	EndOfTrial	A3	F	37	4.8	104	41.9	120	48.5
Synbiotic	EndOfTrial	A4	F	47	4.3	80.8	30.4	94	44.4
Synbiotic	EndOfTrial	A5	M	43	5.3	92.5	27.7	104	29.3
Synbiotic	EndOfTrial	A6	F	42	5.2	91	34.5	108	47.7
Synbiotic	EndOfTrial	A7	F	60	5.7	89.5	37.3	101	45.1
Synbiotic	EndOfTrial	A8	M	40	5.3	78.9	27.3	96	22.6
Synbiotic	EndOfTrial	A9	F	50	5.1	81.6	34	111	NA
Placebo	EndOfTrial	B1	M	51	5.9	133	35.7	123	33
Placebo	EndOfTrial	B10	F	79	5	77.7	27.7	105	40.3
Placebo	EndOfTrial	B11	F	34	4.9	68	24.2	88	NA
Placebo	EndOfTrial	B3	F	45	4.6	76.2	27.7	99	42.8
Placebo	EndOfTrial	B4	F	39	4.9	86.6	26.9	85	38
Placebo	EndOfTrial	B5	F	51	4.6	79.2	30	94	35
Placebo	EndOfTrial	B6	F	39	5.2	100.6	32.8	108	41.4
Placebo	EndOfTrial	B7	F	50	5	93.5	36.5	108	46.1
Placebo	EndOfTrial	B8	M	22	5.3	119	32.3	115	NA

Placebo	EndOfTrial	B9	F	60	5.2	66.3	27.6	86	NA
Treatment	Timepoint	Subject	Gender	Age	HbA1C_percent	Body_mass_kg	BMI_kg_per_m2	WC_cm	Body_Fat_percent
Synbiotic	Baseline	A1	F	48	5.6	66.4	27.5	98	35.6
Synbiotic	Baseline	A10	F	66	5.3	84.8	34.2	106	48.5
Synbiotic	Baseline	A11	M	45	5.3	93	30.3	113	NA
Synbiotic	Baseline	A3	F	37	5.5	113	45.6	127	49.9
Synbiotic	Baseline	A4	F	47	5.1	82.9	31.2	102	45.5
Synbiotic	Baseline	A5	M	43	5.4	97.5	29.2	110	27.6
Synbiotic	Baseline	A6	F	42	5.9	95.2	36.1	108	44.4
Synbiotic	Baseline	A7	F	60	5.7	95.7	39.8	108	44.8
Synbiotic	Baseline	A8	M	40	5.1	88.4	30.6	107	27.8
Synbiotic	Baseline	A9	F	50	5	90	37.5	117	NA
Placebo	Baseline	B1	M	51	8.2	147.4	39.6	129	31.7
Placebo	Baseline	B10	F	79	4.9	78.2	27.9	106	42.3
Placebo	Baseline	B11	F	34	4.8	78.7	28.1	97	NA
Placebo	Baseline	B3	F	45	5.5	81.6	29.6	102	43.3
Placebo	Baseline	B4	F	39	5.3	99.7	30.9	103	42.1
Placebo	Baseline	B5	F	51	5.5	86.8	32.9	95	38
Placebo	Baseline	B6	F	39	4.3	112	36.6	117	41.5
Placebo	Baseline	B7	F	50	4.6	102.9	40.2	118	47.9
Placebo	Baseline	B8	M	22	5.3	117	31.7	114	NA
Placebo	Baseline	B9	F	60	5.2	72.5	30.2	88	NA

Treatment	Timepoint	Subject	Gender	Age	Lean_Mass_kg	Fat_Mass_kg	BMC_kg	BLM.BMC_kg
Synbiotic	EndOfTrial	A1	F	48	40.8	21.98	1.98027	42.7845
Synbiotic	EndOfTrial	A10	F	66	40.9	30.31	1.72035	42.6258
Synbiotic	EndOfTrial	A11	M	45	NA	NA	NA	NA
Synbiotic	EndOfTrial	A3	F	37	52.95	52.37	2.61584	55.5709
Synbiotic	EndOfTrial	A4	F	47	43.96	36.72	1.97749	45.9383
Synbiotic	EndOfTrial	A5	M	43	63.95	27.78	3.06272	67.0143
Synbiotic	EndOfTrial	A6	F	42	46.05	44.27	2.41203	48.4709
Synbiotic	EndOfTrial	A7	F	60	47.37	40.76	2.31609	49.6947
Synbiotic	EndOfTrial	A8	M	40	59.8	18.31	2.95954	62.792
Synbiotic	EndOfTrial	A9	F	50	NA	NA	NA	NA
Placebo	EndOfTrial	B1	M	51	90.64	46.57	4.01039	94.6529
Placebo	EndOfTrial	B10	F	79	44.5	31.47	2.10646	46.6281
Placebo	EndOfTrial	B11	F	34	NA	NA	NA	NA
Placebo	EndOfTrial	B3	F	45	44.44	34.76	2.0451	46.492
Placebo	EndOfTrial	B4	F	39	51.97	33.76	3.03328	55.0122
Placebo	EndOfTrial	B5	F	51	50.61	28.53	2.32471	52.9441
Placebo	EndOfTrial	B6	F	39	58.25	43.03	2.71764	60.973
Placebo	EndOfTrial	B7	F	50	48.9	44	2.56914	51.4396
Placebo	EndOfTrial	B8	M	22	NA	NA	NA	NA
Placebo	EndOfTrial	B9	F	60	NA	NA	NA	NA
Treatment	Timepoint	Subject	Gender	Age	Lean_Mass_kg	Fat_Mass_kg	BMC_kg	BLM.BMC_kg
Synbiotic	Baseline	A1	F	48	41.38	23.93	1.97853	43.3602

Synbiotic	Baseline	A10	F	66	42.5	41.58	1.72548	44.2227
Synbiotic	Baseline	A11	M	45	NA	NA	NA	NA
Synbiotic	Baseline	A3	F	37	54.37	56.73	2.62759	57.0028
Synbiotic	Baseline	A4	F	47	43.64	38.12	1.99988	45.6463
Synbiotic	Baseline	A5	M	43	65.33	26.08	3.08403	68.4227
Synbiotic	Baseline	A6	F	42	50.02	41.79	2.37072	52.3916
Synbiotic	Baseline	A7	F	60	50.13	42.65	2.33551	52.4722
Synbiotic	Baseline	A8	M	40	61.7	24.9	2.96073	64.6943
Synbiotic	Baseline	A9	F	50	NA	NA	NA	NA
Placebo	Baseline	B1	M	51	94.5	45.72	3.94956	98.4542
Placebo	Baseline	B10	F	79	41.6	32.1	2.16976	43.7326
Placebo	Baseline	B11	F	34	NA	NA	NA	NA
Placebo	Baseline	B3	F	45	44.49	35.57	2.08773	46.5816
Placebo	Baseline	B4	F	39	55.81	42.72	2.9912	58.8061
Placebo	Baseline	B5	F	51	51.88	33.17	2.35626	54.2397
Placebo	Baseline	B6	F	39	62.49	46.25	2.67815	65.1701
Placebo	Baseline	B7	F	50	51	49.09	2.44216	53.4179
Placebo	Baseline	B8	M	22	NA	NA	NA	NA
Placebo	Baseline	B9	F	60	NA	NA	NA	NA

Table 3-S1. Body weight (g) and time (days) measurements of mice fed diet supplemented with dairy products.

	0 day	7 day	14 day	21 day	28 day	35 day
Cow Milk	15.1	16.4	17	17.5	18.7	18.8
	14.2	15.9	16.9	17.6	18.1	19.2
	16.5	17.9	19.1	19.3	20.4	18.2
	14.6	15.9	17.1	17	18.2	19.5
	15.1	16.7	17.5	17.6	18.5	18.7
Goat Milk	14.2	15.1	16.5	16.4	17.9	18.9
	12.6	14.6	15.4	15.8	16.3	16.9
	15.6	17.6	17.9	18.3	18.5	18.9
	15	16	16.7	17.1	17	18.1
	15.7	17.1	17.9	18.3	18.7	19.5
Camel Milk	15.5	16.5	17.9	17.9	18.1	18.4
	15.4	16.1	17.7	17	18.1	18.4
	14.5	15.5	16.5	16.3	16.4	16.3
	16.5	17	17.9	17.4	18.2	18.6
	14.2	15.8	16.8	16.5	18.1	18.3
Cow Yogurt	15.5	16.1	17.3	17.1	18.1	18.4
	14.8	16.1	17.1	17.1	17.1	18.2
	14.7	16.3	17.4	18.5	18.7	18.9
	16.9	17.7	18.3	20.2	19.8	20.8
	15.3	16.1	16.3	16.4	16.3	16.9
Goat Yogurt	15.6	17.9	18.6	18.9	18.9	18.9
	15.7	16.1	17.2	17.2	18.1	18.2
	17	17.4	18.1	18.4	18.8	18.9
	17.4	17.4	18.6	18.7	19.8	20.1
	16.8	17.4	18.6	19.1	19.1	19.7
Cow Yogurt Pro	17	18.5	18.7	19.2	20.3	20.9
	16	16.7	17.4	17.5	18.4	18.7
	15.9	16.7	17.3	17.7	18	17.5
	17	18.1	19.4	19.5	20.5	20.7
	15.8	16.5	17.8	17.7	18.6	18.8
Goat Yogurt Pro	17.2	17.9	19	19.2	19.3	20.3
	16.8	17.3	17.7	18.9	19.4	20.1
	17.3	18.9	19.1	19.8	20.3	19.6
	13.6	14.5	15.3	17.1	18.3	18.1
	14.3	16.1	16.9	18.5	20.1	21.1
Cow Cheese	14.8	16.5	16.9	17.5	18.4	19.5

	16.4	17	17.7	18.5	18.4	18.6
	15.6	15.7	16.3	17.2	17	17.4
	16.6	18.5	18.2	18.6	19.2	19.3
	15.3	16.9	16.3	17.1	17.8	19
Cow Cheese Pro	15.1	15.5	16.5	17.3	17.3	17.9
	16.7	17.5	18.1	18.2	18.1	18.8
	14.5	15.3	16.6	17.7	17.5	18.3
	16.4	16.9	17.2	18.2	18.6	18.8
	17	17.5	18.7	18.9	18.6	19.6
Ctrl	15.7	16.2	16.5	17.6	16.7	16.3
	15.4	15.4	17.2	17.4	17.3	16.8
	16.6	17.4	17.8	19.1	18.1	17.8
	15.5	16.4	17.3	18.4	18.4	18
	15.8	16.7	17.3	18.3	18.2	17.6