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Table grape consumption reduces adiposity and markers of hepatic lipogenesis and alters gut microbiota in butter fat-fed mice

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Abstract

Our objective was to determine if consuming table grapes reduces adiposity and its metabolic consequences and alters gut microbiota in mice fed a high fat (HF), butter-rich diet. C57BL/6J mice were fed a low fat (LF) diet or HF diet with 3% or 5% grapes for 11 weeks. Total body and inguinal fat were moderately, but significantly reduced in mice fed both levels of grapes compared to their controls. Mice fed 5% grapes had lower liver weights and triglyceride levels, and decreased expression of glycerol-3-phosphate acyltransferase (*Gpat1*) compared to the 5% controls. Mice fed 3% grapes had lower hepatic mRNA levels of peroxisome proliferator-activated receptor gamma 2, sterol-CoA desaturase 1, fatty-acid binding protein 4, and *Gpat1* compared to the 3% controls. Although grape feeding had only a minor impact on markers of inflammation or lipogenesis in adipose tissue or intestine, 3% grapes decreased the intestinal abundance of sulfidogenic *Desulfobacter* spp., and the *Bilophila wadsworthia*-specific dissimilatory sulfite reductase gene (*dsrA-Bw*), and tended to increase the abundance of the beneficial bacterium *Akkermansia muciniphila* compared to controls. Additionally, *Bifidobacterium*, *Lactobacillus*, *Allobaculum*, and several other genera correlated negatively with adiposity. *Allobaculum* in particular was increased in the LF and 3% grapes groups compared to the HF-fed controls. Notably, grape feeding attenuated the HF-induced impairment in epithelial localization of the intestinal tight junction protein zonula occludens. Collectively, these data indicate that some of the

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adverse health consequences of consuming a HF diet rich in saturated fat can be attenuated by table grape consumption.

Keywords

grapes; microbiota; intestines; obesity; steatosis

1. Introduction

Currently, 36% of adults and 17% of youth in the United States have been diagnosed as obese [1]. Obesity is also a global health condition affecting more than 10% of adults worldwide [2]. Of growing concern is the association between the rise in obesity and chronic inflammatory conditions such as type 2 diabetes, hypertension, and cardiovascular disease [3]. Physiologically, obesity is the result of an expansion of white adipose tissue (WAT) which typically elicits inflammatory signals involved in the recruitment of macrophages and other immune cells into the WAT. This results in an increase in circulating and tissue levels of proinflammatory cytokines, chemokines, and adipokine [4]. While increased tissue levels of these proinflammatory agents perpetuates the inflammatory cycle, those released systemically impair glucose disposal and lipid metabolism that contribute to the development of metabolic diseases. However, the exact mechanisms which initiate WAT inflammation resulting from diet-induced obesity remain unclear.

Gut microbes have received much attention due to their potential involvement in the development of obesity [5], chronic inflammation [6–8], and insulin resistance [9]. Diets high in fat, particularly those rich in saturated fatty acids found in milk fat [10], have been implicated in the reduction of gut barrier-protecting bacteria as well as an increase in the abundance of deleterious bacteria including sulfidogenic bacteria [reviewed in 11]. A correlation exists between the extent of these changes in microbiota and the degree of obesity and insulin resistance in test subjects [12]. Furthermore, the main byproduct of sulfidogenic bacteria like *Bilophila wadsworthia* and other members of the *Desulfovibrionaceae* family is hydrogen sulfide, a proinflammatory and genotoxic gas that is positively correlated with development of ulcerative colitis, gut inflammation, irritable bowel syndrome, and colon cancer [10, 13, 14].

In contrast, changes to the intestinal microbiome can have beneficial effects. For instance, research with animal models has demonstrated that elimination of gut microbes [5–9, 11, 12, 15] or inoculation with specific pre- (e.g., non-digestible sugars, fiber, or polyphenols) or probiotics (e.g., *Lactobacillus acidophilus*, *Bifidobacterium* spp., *Akkermansia muciniphila*, *Clostridium trybutyricum*) [reviewed in 11, 16–19] can attenuate obesity or metabolic dysfunction. Polyphenols found in fruits and vegetables [20–22] are of particular interest as they are poorly absorbed in the upper gastrointestinal tract, and thus persist in the distal small intestine, cecum, and colon [23], where they may influence microbial taxa and their metabolites [24]. In addition, the anti-inflammatory, anti-oxidant, or anti-microbial actions of polyphenols have been reported to positively influence gut microbes and inflammation [reviewed in 25].

plus 5% powdered grapes (HF-5G) and high fat plus 5% sugar (HF-5S). The HF diets consisted of approximately 3% energy from soybean oil and 31% energy from butter. Thus, the HF diets were rich in fat, especially saturated fat (i.e. 20.3% of energy), and mimicked the average calories from fat in an American-type diet (i.e., 34% of energy). The lyophilized (i.e., powdered) table grapes, kindly provided by the California Table Grape Commission consisted of a mixture of red, green, and black seeded and seedless grapes. The 3% and 5% dietary levels of grapes were comparable to 9 and 15 human servings (1 serving is equivalent to 3/4 cup of whole grapes) of grapes, respectively. The HF-3S and HF-5S diets consisted of a mixture of fructose and glucose to control for the natural sugar content of the powdered grape diets. Diets were prepared by Research Diets (New Brunswick, NJ, USA) and stored at -20°C until use. Detailed composition of the diets is provided in Supplemental Table 1.

2.3. Intraperitoneal glucose tolerance tests (GTT)s and fasting insulin levels

Intraperitoneal (i.p.) GTTs were performed on weeks 3, 6, and 9 on non-anesthetized mice. Mice were fasted for 8 h and given an i.p. injection of glucose (i.e., 20% solution at 1 g/kg body weight). Blood from the tail vein collected at baseline and 5, 15, 30, 60, and 120 minutes post-i.p. glucose injection was used to quantify glucose levels using a Bayer Contour blood glucose monitor and strips (Bayer Healthcare, Tarrytown, NY, USA). Plasma insulin levels were detected using an ultrasensitive mouse insulin kit (Crystal Chem, Inc, Downers Grove, IL). The homeostasis model assessment method (HOMA) for insulin resistance (IR) was used employing the following formula: $[\text{fasting insulin concentration (ng/ml)} \times 24 \times \text{fasting glucose concentration (mg/dl)}] / 405$ [16].

2.4. Body fat measurements via Dual X-Ray Absorptiometry (DEXA)

Percent body fat was measured using DEXA on a GE Lunar Prodigy Advanced System (GE Healthcare, Milwaukee, WI) at weeks 5 and 10. During the measurement, mice were lightly anesthetized with isoflurane using a SomnoSuite Small Animal Anesthesia System with Integrated Digital Vaporizer isoflurane system. Measurements were taken in duplicate to reduce the possibility of error and values expressed are an average of the two measurements.

2.5. Tissue collection

After 11 weeks of dietary intervention, mice were fasted for 8 h and euthanized via isoflurane-induced anesthesia followed by decapitation. Plasma was collected at the time of harvest. Four WAT depots were collected; epididymal, mesenteric, inguinal, and retroperitoneal. Additionally, livers were harvested and intestinal mucosa and digesta were collected from the duodenum, jejunum, ileum, cecum, and proximal and distal colon. Weights of the WAT depots and liver were recorded and all collected samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until analysis.

2.6. Liver and serum triglyceride (TG) levels and Oil-Red-O staining

Liver TG content was measured as previously described [31]. Plasma TG content was determined using a commercial assay from Thermo Scientific and was conducted following the manufacturer's protocol (Infinity TG assay #TR22421 and TG standards #TG22923;

Norcross, GA). For Oil-Red-O staining, frozen liver tissues were cut at 5 μm , and mounted on slides. The sections were fixed with 10% formalin for 10 minutes and then the slides were washed with deionized water for 5 minutes. Fixed tissues were then rinsed with 60% isopropanol for 5 minutes. To prepare Oil-Red-O stock, 0.5 g of Oil-Red-O (Sigma-Aldrich, O0625) was mixed with 100 mL of isopropanol. To prepare Oil-Red-O working solution, 30 mL of Oil-Red-O stock was mixed with 20 mL of distilled water and filtered using a 0.24 μm vacuum filter. Samples were submerged in the working solution for 15 minutes, briefly rinsed with 60% isopropanol, and then rinsed with deionized water for 30 seconds before imaging.

2.7. RNA extraction and qPCR

Adipose and intestinal samples were homogenized in QIAzol reagent and total RNA was extracted using QIAgen mini lipid kit obtained from Qiagen (Valencia, CA). For hepatic samples, a QIAgen mini universal kit from Qiagen was used. The quality and concentration of RNA were examined using absorbance at 260 nm and integrity determined using the absorbance ratio of 260/280 on a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA was created by reverse transcription using 1 μg of RNA and a high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocols. qPCR was performed in a 7500 FAST Real Time PCR system (Applied Biosystems). The expression of different genes related to inflammation, lipogenesis, and lipolysis in WAT depots and liver were measured using Taqman Gene expression assays purchased from Applied Biosystems. TATA-binding protein (*Tbp*) was the endogenous reference gene utilized for all assays and fold differences in gene expression were calculated as $2^{-\text{Ct}}$.

2.8. Immunoblotting

Immunoblotting was conducted as previously described [31] using primary antibodies at 1:1000 dilutions for stearyl-CoA desaturase-1 (#2283S; SCD1, Cell Signaling), carnitine palmitoyltransferase 1A (#12252S; CPT1a, Cell Signaling), PPAR γ (#2443S; Cell Signaling Technology, Inc., Danvers, MA), β -actin (#4967; Cell Signaling), PPAR α , (#sc9000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), glycerol-3-phosphate acyltransferase (#sc382257; GPAM, Santa Cruz), and sterol regulatory element-binding protein 1C (#sc366; SREBP1c, Santa Cruz). Adipocyte fatty acid binding protein (aP2) was kindly provided by Dr. David Bernlohr (U. of Minnesota) and used at a 1:10,000 dilution. Horseradish peroxidase-conjugated secondary antibodies were probed for 2 h at room temperature at 1:1000 dilutions. Blots were exposed to a chemiluminescence reagent and X-ray films were developed using a SRX-101A Konica Minolta film developer.

2.9. Barrier function in ileum

The localization of the tight junction protein zonula occludens-1 (ZO-1) was determined in ileal mucosa samples that had been embedded in Tissue-Tek Cyro-OCT compound, sliced in 5 μm sections, fixed in methanol at -20°C , and incubated with a polyclonal rabbit anti-ZO-1 antibody as previously described [32]. Three to four samples of each group were analyzed by Image J software. Each photo was corrected by its own background and then adjusted by DAPI.

2.10. PCR amplification of 16S rRNA and functional gene targets

Genomic DNA extraction and qPCR experiments were conducted as previously described [32]. 16S rRNA gene-specific primers were used to target specific bacterial genera; *A. muciniphila*, *B. wadsworthia* (F-AAGTCCTTCGGGGCGAGTAA) (R-ATCCTCTCAGACCGGCTAC), *Desulfovibrio* spp. (DSV), *Desulfobulbus* spp. (DBB), *Desulfobacter* spp. (DSB), and *Desulfotomaculum* spp. (DFM) [32]. The *B. wadsworthia*-specific dissimilatory sulfite reductase (*dsrA*-Bw) [20,32], which encodes an enzyme that catalyzes a step in the reduction of sulfite to hydrogen sulfide, was targeted to measure the abundance of this member of the *Desulfovibrionaceae* family. Four functional genes utilized by *Fusobacterium nucleatum* in the fermentation of cysteine were measured; i.e., two homologues of L-cysteine desulhydrase (FN0625 and FN1220), Cystathionine- β synthase (FN1055), and L-methionine- γ -lyase (FN1419). Standard curves were constructed using cloned 16S rRNA and functional genes or amplified PCR product.

2.11. Sequencing of 16s rRNA gene using Illumina Mi-Seq platform

To assess bacterial community structure of cecal mucosa, primers specific for 16S rRNA V4–V5 region (Forward: 338F: 5'-GTGCCAGCMGCCGCGGTAA-3' and Reverse: 806R: 5'-GGACTACHVGGGTWTCTAAT-3') that contained Illumina 3' adapter sequences as well as a 12-bp barcode were used. Sequences were generated by an Illumina MiSeq DNA platform at Argonne National Laboratory and analyzed by the program Quantitative Insights Into Microbial Ecology (QIIME) [33]. Operational Taxonomic Units (OTUs) were picked at 97% sequence identity using open reference OTU picking against the Greengenes database (05/13 release) [34]. OTUs were quality filtered based on default parameters set in the open-reference OTU command in QIIME and sequences were rarified to an equal sampling depth of 15000 reads per sample. Representative sequences were aligned via PyNASt [33], taxonomy assigned using the RDP Classifier [35], and a phylogenetic tree was built using FastTree [36]. Beta Diversity was represented by measuring UniFrac distances calculated using both weighted and unweighted algorithms and visualized with PCoA plots generated in Emperor (Supplemental Fig 1). Prior to statistical analyses, OTUs occurring in less than 50% of samples were filtered from the OTU table. Significant changes in OTU abundance were assessed using Kruskal Wallis test (FDR correction $p < 0.05$). Multivariate statistical tests performed included ADONIS, ANOSIM, and PERMANOVA tests [33]. Spearman correlations and Principal Component Analysis (PCA) were run using MATLAB software.

2.12. Statistical analysis

Data were analyzed using a one-way ANOVA and Student's *t* test to compute individual pairwise comparisons of means ($p < 0.05$). We also used Bonferroni's posthoc test to perform specific comparisons where appropriate. Analyses were conducted using the JMP software program version 10.0 for Windows (SAS, Cary, NC). Relative abundances generated from 16s rRNA sequencing were analyzed in GraphPad Prism Version 6 using ANOVA followed by Dunn's test for multiple comparisons. Data are expressed as means + S.E.M.

3. Results

3.1. Grape consumption decreases body fat

Body weight gains and energy intakes were greater in all HF-fed mice compared to the LF control mice (Table 1). Mice fed powdered grapes (HF-3G, HF-5G) had similar body weights and energy intakes compared to their HF-sugar controls (HF-3S, HF-5S; Table 1). Mice fed the HF-sugar control diets had greater body fat percentage and total WAT depot weights compared to the LF controls (Fig. 1). Mice fed the HF-3G diet had lower percent body fat at week 5 and mice fed powdered grapes at both levels had lower body fat percentages and inguinal fat depot weights at week 10 compared to their respective HF-sugar controls (Fig. 1). The HF-3G diet had lower total WAT depot weights compared to HF-3S controls (Fig. 1).

3.2. GTTs, fasting glucose, insulin, and TG levels, and HOMA-IR scores unaffected by grapes

To assess the impact of grape consumption on insulin resistance and glucose sensitivity, GTTs were conducted at weeks 3, 6, and 10, and fasting plasma glucose and insulin and HOMA-IR were measured at week 11. Mice consuming the HF-sugar control diets had impaired GTTs at all three time points compared to the LF controls (Fig. 2). However, consuming grapes did not significantly improve GTT, fasting insulin, glucose, serum TG levels, or HOMA-IR scores.

3.3. Grape consumption lowers hepatic TG levels, and the expression of several lipogenic genes

Liver tissues were analyzed to assess the impact of grape consumption on liver TG levels and markers of lipogenesis and fatty acid oxidation. Mice consuming the HF-sugar control diets had greater liver weights, TG levels (HF-5S only), visual lipid staining, and mRNA levels of the lipogenic genes *Ppar γ 2*, *Scd1* (HF-5S only), *Srebp1c*, cluster of differentiation 36 (*Cd36*; HF-5S only), and glycerol-3-phosphate acyltransferase 1 (*Gpat1*; HF-5S only) compared to the LF-fed mice (Fig. 3). Mice fed the HF-3G diet had decreased mRNA levels of *Ppar γ 2*, *Scd1*, fatty acid binding protein 4 (*Fabp4*), and *Gpat1* compared to the HF-3S controls. Mice fed the HF-5G diet had decreased TG levels and mRNA levels of *Gpat1* compared to the HF-5S controls. None of the genes associated with fatty acid oxidation (e.g., *Ppara*, *Cpt1a*, acyl-CoA oxidase 1 (*Acox1*)) were impacted by grape feeding. Grape consumption did not impact the protein levels of *Ppar γ* , *Scd1*, *Ppara*, *Fabp*, *Cpt1a*, or *Gpam* (data not shown) or influence the ratio of liver weight to body weight..

3.4. Grape consumption differentially impacts WAT genes associated with inflammation and lipid metabolism

To determine whether insulin resistance caused by HF-feeding was due to WAT inflammation, inguinal (subcutaneous), epididymal (visceral), and mesenteric (visceral) WAT mRNA levels for several proinflammatory genes (i.e., cluster of differentiation 11c (*Cd11c*), epidermal growth factor-like module containing mucin-like hormone receptor 1 (*Erm1*; F4/80 human orthologue), monocyte chemoattractant protein 1 (*Mcp1*), *Tnfa*, Toll-

like receptor 4 (*Tlr4*), and interleukin 6 (*Il6*) were measured. In epididymal WAT, mice fed the HF-sugar controls had increased mRNA levels of *Tnfa* (HF-5S), *Cd11c* (HF-5S), *Il6*, and *Mcp1* (HF-5S) compared to the LF controls (Fig. 4A). Grape feeding did not impact the expression of these genes. In inguinal depot WAT, only mice fed the HF-5S diet exhibited increased inflammatory gene expression (i.e., *Mcp1*), which was decreased in mice fed the HF-5G diet (Fig. 4B). In mesenteric WAT, inflammatory gene expression was not increased by HF-feeding.

To determine if the reduction in adiposity by grape consumption was due to alterations in the expression of genes associated with fat synthesis or oxidation, epididymal and inguinal WAT were analyzed for mRNA markers of: (i) lipogenesis (i.e., *Ppar γ 2*, *Srebp1c*, *Scd1*, acylglycerol-3-phosphate-O-acyltransferase 2 (*Agpat2*), fatty acid synthase (*Fas*), acetyl-CoA carboxylase (*Acc*), perilipin 1 (*Plin1*), *Gpat1*); (ii) lipolysis (i.e., adipose TG lipase (*Atgl*); and (iii) beta-oxidation (i.e., *Acox1*, *Cpt1b*, *Ppara*). In epididymal WAT, the mRNA levels of the lipogenic genes *Ppar γ 2*, *Agpat2*, and *Scd1* were higher in the HF-5S group compared to the LF group, and similar to the HF-grape groups (Fig. 4C). In inguinal WAT, the mRNA levels of the lipogenic genes *Srebp1c* (HF-5S), *Scd1*, *Agpat2* (HF-5S), *Fas* (HF-5S), and *Gpat2* (HF-5S) were higher in the HF-sugar controls compared to the LF-controls (Fig. 4D). Mice fed the HF-5G diets exhibited lower mRNA expression of *Agpat2* compared to HF-5S controls. The mRNA levels of the fatty-acid oxidizing genes *Ppara* and *Cpt1b*, and the lipolytic gene *Atgl* (HF-5S) were lower in the HF-sugar controls compared to the LF control, and similar to the HF-grape groups (Fig. 4D). The mRNA levels of *Plin1*, *Acc*, *Acox1*, uncoupling protein1, or cytochrome8b in epididymal or inguinal WAT were not significantly impacted in mice fed the grape diets (data not shown).

3.5. Minimal influence of HF-feeding and grape consumption on markers of intestinal inflammation and barrier function

Given the reported adverse effects of consuming saturated fats [reviewed in 11], particularly from milk fat [10], on intestinal health, and potential prebiotic impact of grapes, we measured the effects of our diets on markers of intestinal inflammation and barrier function. For inflammatory status, mRNA expression of *Cd11c*, *Erm1*, *Mcp1*, *Tnfa*, *Tlr4*, and *Il6* was quantified in ileal and distal colonic mucosa and the activities of duodenal alkaline phosphatase and ileal myeloperoxidase were measured. Surprisingly, the only proinflammatory gene increased in the ileal mucosa of HF-sugar-fed mice was *Tnfa*, which was similar to the HF-5G group (data not shown). None of the proinflammatory genes measured in the colonic mucosa were increased by HF-sugar feeding or grape consumption (data not shown). Similarly, the activities of alkaline phosphatase and myeloperoxidase, enzymes associated with increased levels of lipopolysaccharide and neutrophils, respectively, were not influenced by HF-sugar feeding or grape consumption (data not shown).

To assess intestinal barrier function, the mRNA levels of the tight junction proteins zonula occludens (*Zo1*), claudin-1, and occludin-1 and the localization of ZO-1 at the apical surface of the ileal epithelium were measured. Although the ileum mucosal gene expression of these tight junction proteins was not impacted by the diets (data not shown), the localization of

ZO-1 was impaired in the HF-sugar control diets compared to the LF controls, and improved by grape feeding, particularly the HF-3G diet (Fig. 5).

3.6. 16s rRNA sequencing of the gut microbiota reveals genera associated with body fat percentage and inguinal fat pad weight

Next, we measured the effects of our diets on the abundance of several mucosal sulfidogenic bacteria (i.e., DBB, DSB, DFM, DSV, and *B. wadsworthia*) or their gene products (i.e., *B. wadsworthia* specific *dsrA-Bw*, *Fusobacterium nucleatum* functional genes including two L-cysteine desulfohydases (*FN0625* and *FN1220*) cystathionine- β synthase (*FN1055*), and L-methionine- γ -lyase (*FN1419*). Although HF-feeding did not increase the abundance of any of these genes associated with sulfur metabolism in ileal or colonic mucosa compared to LF control mice (data not shown), the abundance of DSB and *dsrA-Bw* was lower in ileal mucosa of mice consuming the HF-3G diet compared to mice consuming the HF-3S diet (Fig. 6A).

We also measured the abundance of *A. muciniphila*, a mucin-degrading bacteria associated with prebiotic-mediated reduction in obesity [37]. Although not statistically significant, mice consuming grapes tended to have increased levels of *A. muciniphila* in the colonic digesta (HF-3G) and proximal colonic mucosa (HF-5G) compared to their HF-sugar controls (Fig. 6B).

3.7. Sequencing of the 16s rRNA gene reveals alterations in microbial structure and relationships to adiposity

In order to better understand the impact of grape feeding on gut bacterial community structure, an untargeted approach was used by sequencing the 16s rRNA gene in cecal mucosal samples. Sequencing was performed on an Illumina MiSeq platform using primers targeting the V4–V5 region of the 16s rRNA gene. Data were analyzed using QIIME 1.8 Software [33]. Alpha Diversity analyses revealed that the HF-5G or HF-5S diets reduced observed species compared to HF-3G ($p = 0.0002$) or HF-3S diets ($p < 0.0001$), respectively (Fig. 7). Notably, the HF-3S diet reduced observed species compared to the HF-3G diet ($p = 0.0026$), but there was no difference in observed species in HF-5G or HF-5S groups. These results suggest that elevated sugar content may result in decreased alpha diversity or membership of the gut microbiota. PCA of weighted UniFrac distances did not reveal obvious differences in beta diversity or community structure across diet groups, but there was clear separation between the LF group and HF groups based on unweighted UniFrac distances (Supplemental Fig. 1). To further interrogate differences between diet groups, multivariate statistical tests including ADONIS, ANOSIM, and PERMANOVA were conducted. Significant differences were found among unweighted and weighted UniFrac distances across diet groups (Table 2). Subsequently, OTUs that were not represented in up to 50% of the samples were removed to reduce noise from low abundance OTUs. Next, a Kruskal Wallis test was performed to determine significant differences in relative abundance of taxa between groups. Here, it was found that several bacterial taxa were significantly altered based on diet (Fig. 8). While many genera were reduced in the HF diets compared to the LF diet, some were selectively increased in HF-3G group such as *Ruminococcus* and *Anaeroplasm* in the Firmicutes and Tenericutes phylum, respectively (Fig. 8).

To assess differences between specific diet groups, PCA was conducted [38] and clear clustering was evident between the LF and HF groups (Fig. 9A) and between the HF-3G and HF-3S group (Fig. 9B). PCA between the LF and HF groups revealed that ~30% of the variance in relative bacterial abundance between the two groups is explained with Principal Component 1 (PC1). Principal component 2 (PC2) explained an additional 20% of the variance between groups and was most strongly correlated with *Bacteroides* and S24-7. PCA between the HF-3G and HF-3S groups revealed that ~60% of the variance in relative microbial abundance between the two groups was explained by PC1. PC1 was strongly correlated with S24-7 and *Clostridiales* (Fig. 9B). PC2 explained ~20% of the variance and exhibited a strong correlation with *Akkermansia*; however this was positively associated with the HF-3S group. These data indicate that most variance between the LF and HF-3G was explained by the abundance of *Bacteroides* in the LF group. Intriguingly, S24-7 also explained the variance between the HF-3G and HF-3S groups, as it was positively correlated with HF-3G compared to HF-3S. These data may indicate that S24-7 is associated with improved metabolic profile of the LF and HF-3G groups.

Lastly, Spearman Correlation analysis revealed significant negative correlations between body fat percentage and several genera (Fig. 10, Table 3) with the most profound being *Bifidobacterium* ($p = 0.0001$), a butyrate producer, and others including *Lactobacillus* ($p = 0.0221$) and *Allobaculum* ($p = 0.034$; Fig 10, Table 3). These particular genera were similarly correlated with inguinal fat pad weight. *Clostridiales* of the Firmicutes phylum was positively correlated with body fat percentage ($p = 0.0065$). Interestingly, a heatmap made from the taxa that were significantly correlated with body fat percentage, revealed that *Allobaculum* belonging to the Tenericutes phylum was increased in the LF and HF3G groups (Fig. 10). Little is known regarding the functional role of *Allobaculum* in the gut ecosystem and is currently being investigated for its potential association with a lean phenotype and improved metabolic health.

4. Discussion

Our data demonstrate that consuming table grapes (i.e., 3–5%, w/w; equivalent to 9–15 human servings) modestly, but significantly attenuates the accumulation of body and liver fat in mice fed a HF diet rich in butter compared to control mice. However, these lipid-lowering effects of grapes were not associated with significant improvements in glucose tolerance or markers of inflammation in intestinal mucosa or WAT. Notably, the impaired localization of the intestinal tight junction protein ZO-1 in HF-fed mice was improved by grape consumption. Abundance of *Desulfobacter* spp, and the *B. wadsworthia*-specific dissimilatory sulfite reductase gene were decreased by grape consumption (HF-3G), and the beneficial bacterium *A. muciniphila* tended to be in greater abundance in the colonic mucosa (HF-5G) or digesta (HF-3G) of grape fed mice compared to their respective controls. Additionally, via 16s rRNA sequencing analysis, significant differences across groups were observed in the relative abundance of S24-7 and *Akkermansia*, which were found to be negatively correlated with HF compared to the LF diet. Intriguingly, correlation analysis showed a strong negative correlation between *Bifidobacterium* and body fat percentage and inguinal fat pad weight. Also evident was an increase in *Allobaculum* in the LF and HF-3G groups. This bacterium has recently been associated with lean phenotypes in several reports

[39–41]; however the mechanism establishing this link is unknown. Taken together, these data indicate that consumption of California table grapes moderately attenuates adiposity and steatosis that are positively correlated with a marker of intestinal integrity and changes in several species of gut microbes in mice fed a HF, butter-rich diet.

4.1. Influence of dietary polyphenols on adiposity, steatosis, and glucose tolerance

Many studies have demonstrated that consuming diets rich in calories from fat, particularly saturated fat, and sugars promote obesity and its metabolic complications [reviewed in 11]. The ability of foods rich in polyphenols, including grapes, to prevent obesity-mediated inflammation or related disorders has been demonstrated [reviewed in 25]. For example, grape seed procyanidin extract supplementation reduces body weight gain and WAT mass in hamsters fed a HF diet [42]. Additionally, mice fed a HF diet supplemented with muscadine grape phytochemicals rich in anthocyanins had decreased body weights, less lipid accumulation in the liver, and improved glucose tolerance compared to HF controls [43].

While several studies demonstrate anti-inflammatory and anti-obesity effects of grapes or their extracts, the mechanisms by which these effects occur are less clear. Anthocyanins are one of the most abundant phytochemicals in grapes [31] and therefore may be responsible for mediating the reductions in adiposity and steatosis. For example, anthocyanins purified from purple sweet potatoes attenuated hepatic lipid accumulation via the activation of adenosine monophosphate-activated protein kinase and decreased expression of SREBP1 and its downstream target genes in mice fed a HF diet [44]. In the present study, powdered grape supplementation at 3% and 5% modestly reduced body weight and fat gain and hepatic lipid accumulation, but surprisingly did not significantly affect the expression of *Srebp1c* and *Fas*. Although we discovered that the expression of several hepatic genes associated with lipogenesis (e.g., *Pparγ*, *Scd1*, *Fabp4*, *Gpat1*) in liver were dampened in mice consuming the HF-3G compared to the HF-3S controls, the amount of protein encoded by these genes was not lowered by grape feeding. However, we did not measure the enzymatic activity of these proteins, and thus do not know if they were impacted by grape consumption.

4.2. Relationship between dietary fat, sulfite-reducing bacteria, barrier function, and inflammation

Devkota et al. [10] demonstrated that feeding a milk-fat-based diet similar in amount and composition to the western diet (i.e., 37% kcals from fat) to IL-10 knockout mice decreased gut barrier-protecting bacteria and increased the sulfite-reducing bacterium *B. Wadsworthia* and intestinal markers of inflammation consistent with inflammatory bowel disease [10]. This outcome was associated with a pro-inflammatory T helper type immune response and increased colitis. Notably, taurine-conjugated bile salts caused these inflammatory responses, in part, due to their high sulfur content, which stimulates sulfite-reducing bacteria like *B. wadsworthia*. In a parallel 3 week study, these authors fed C57BL/6J mice the same HF diet [10]. Consistent with the IL-10 knockout mice, C57BL/6J mice fed the milk fat diet exhibited increased abundance of the sulfidogenic bacterium *B. wadsworthia* and the *B. wadsworthia*-specific dissimilatory sulfite reductase gene *dsrA*-Bw. Milk fat-fed C57BL/6J mice also had a higher abundance of Bacteroidetes and a lower abundance of Firmicutes

compared to the LF-fed mice [10]. However, milk fat-fed C57BL/6J mice did not present overt colitis as did the IL-10 knockout mice [10]. In the present study, we did not observe any increases in the abundance of sulfidogenic bacteria-related genes in the HF-fed controls compared to the LF-fed mice, although *dsrA-Bw* and DSB were present in lower abundance in the ileum mucosa of HF-3G mice compared to the HF-3S control group. This decreased abundance of sulfidogenic bacteria correlated with improved localization of the ileal tight junction protein ZO-1. Although markers of intestinal inflammation were not significantly increased by the milk fat-based diet or decreased by grape feeding, the expression levels of several inflammatory genes in visceral epididymal WAT (i.e., *Tnfa*, *Cd11c*, *Mcp1*) and subcutaneous inguinal WAT (i.e., *Mcp1*) were increased by HF feeding (Fig. 4A), and HF-5G consumption tempered *Mcp1* expression in inguinal WAT compared to its control HF-5S.

These results are comparable to data showing that HF-feeding selectively increases the abundance of a specific type of bacteria that are associated with intestinal inflammation [45]. Apolipoprotein A-I (*apoA-I*) knockout mice, which present impaired glucose tolerance and increased adiposity, and wild type mice were fed a very HF diet (i.e., ~60% kcals from lard) for 25 weeks. HF-fed mice had increased abundance of *Desulfovibrionaceae*, a family of sulfate/sulfite reducing bacteria that produce hydrogen sulfide, a genotoxic gas that causes barrier dysfunction and endotoxemia [46]. Consistent with these data, we previously showed that C57BL/6J mice fed a very HF diet (i.e., ~60% kcals from lard) for 20 weeks had increased levels of three types of sulfidogenic bacteria in colonic mucosa, impaired localization of ZO-1, and increased mRNA levels of markers of macrophage infiltration in intestinal mucosa and WAT compared to LF-fed mice [32]. Collectively, these data demonstrate that consuming diets enriched with lard or butter, two fat sources containing high levels of saturated fatty acids, increases the abundance of sulfidogenic bacteria associated with impairment of intestinal barrier function and contribute to systemic inflammation.

4.2. Relationship between dietary fat, *A. muciniphila*, barrier function, and inflammation

A. muciniphila is a commensal, mucin-degrading bacterium that plays a role in preventing the development of diet-induced obesity [37]. Under normal conditions, these bacteria represent 3–5% of the gut microbial population in humans. However, HF-feeding reduces the colonic abundance of these bacteria in mice 100-fold, and oligofructose prebiotic treatment prevents this loss [37]. Oligofructose-mediated increase in *A. muciniphila* was linked to decreased metabolic endotoxemia and markers of inflammation in WAT. Furthermore, supplementation of C57BL/6J mice fed a HF diet with *A. muciniphila* for 4 weeks lead to a decrease in adiposity, gut barrier dysfunction, metabolic endotoxemia, glucose intolerance, insulin resistance, and *Cd11c* expression in WAT, and improved mucus thickness along the epithelium compared to control mice [37]. Consistent with these data demonstrating that oligofructose prebiotic treatment attenuates a HF diet-induced reduction of *A. muciniphila*, our data indicate that supplementing HF butter-rich diets with 5% California table grapes modestly increases the abundance of *A. muciniphila* in proximal colon mucosa compared to the HF-sugar controls. This increase in *A. muciniphila* was positively associated with improved localization of ZO-1 in the apical area of the ileal

epithelium compared to HF control mice. Furthermore, based on our 16S rRNA sequencing data of the cecal mucosa, abundance of the genus *Akkermansia* was positively associated with mice fed a LF diet, further supporting a relationship with a lean phenotype.

4.4. Limitations and unanswered questions

The current study did not achieve the anticipated increases in intestinal and systemic inflammation in young C57Bl/6J mice fed a HF diet rich in milk-fat as demonstrated in IL-10 knockout mice fed milk-fat [10] or in *apo A-I* knockout mice fed lard [44]. Perhaps feeding mice extremely HF diets (e.g., 60% kcals from lard) is necessary to instigate intestinal and systemic inflammation in relatively short term studies [11, 30]. The current study sought to provide a more physiological approach to achieve diet-induced obesity accompanied by systemic and intestinal inflammation. However, the use of this more physiological model (i.e., an amount of fat and sugar calories similar to the average American diet; 34% and 16%, respectively) for the time span of 11 weeks may not have been aggressive enough in this mouse model to elicit a robust inflammatory response; especially because mice were started on the diet at a relatively young age (e.g., 5-weeks of age). Moreover, there were differential effects between the two grape diets, depending on the outcome measured, that lack explanation by the authors. Furthermore, we do not know which compound(s) in table grapes reduced adiposity and steatosis. Therefore, future studies should examine the effect of different grape fractions (e.g., phytochemical, fiber, or sugar-rich fraction) or specific polyphenols on the development of obesity, steatosis, and inflammation using a fat composition that is more typical of the average American diet (i.e., a mixture of animal and vegetable fats consisting of beef tallow, lard, milk fat, shortening, and vegetable oils [47]), instead of mostly butter fat.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Acox

acyl-CoA oxidase

Agpat

acylglycerol-3-phosphate-O-acyltransferase

aP2

adipose fatty acid binding protein

Atgl

adipose triglyceride lipase

AUC

area under the curve

B. wadsworthia

Bilophila wadsworthia

Cd36

cluster of differentiation 36

Cd11c

cluster of differentiation 11c

CPT

carnitine palmitoyltransferase

DEXA

dual-energy X-ray

DBB

Desulfobulbus spp

DFM

Desulfotomaculum spp

DSB

Desulfobacter spp

dsrA-Bw

B. wadsworthia specific dissimilatory sulfite reductase

DSV

Desulfovibrio spp

EPI

epididymal

***Erm1* (F4/80 human orthologue)**

epidermal growth factor-like module containing mucin-like hormone receptor 1

Fas

fatty acid synthase

Fabp4

fatty acid binding protein 4

Gpat

glycerol-3-phosphate acyltransferase

GTT

glucose tolerance test

HF

high fat

HOMA-IR

homeostasis model assessment method for insulin resistance

ING

inguinal

LDL

low density lipoprotein

IL

interleukin

LF

low fat

MCP

monocyte chemoattractant protein

NF- κ B

nuclear factor kappa B

OTU

operational taxonomic units

PCA

primary component analysis

PPAR

peroxisome proliferator activated receptor

QIIME

quantitative insights into microbial ecology

Scd1

stearoyl-CoA desaturase 1

Srebp

sterol regulatory element binding protein

Tbp

TATA-binding protein (*TBP*)

TG

triglyceride

Tlr4

toll-like receptor 4

Tnf

tumor necrosis factor

WAT

white adipose tissue

ZO-1

zonula occludens

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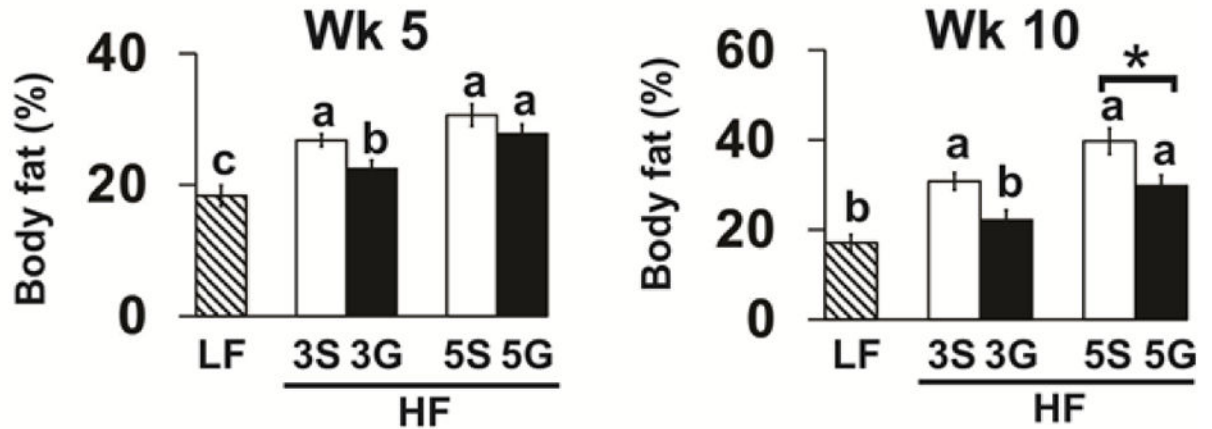
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Appendix A. Supplementary data

Supplementary data (1 table, 1 color figure) to this article can be found online.

A- Body Fat %



B- WAT (g)

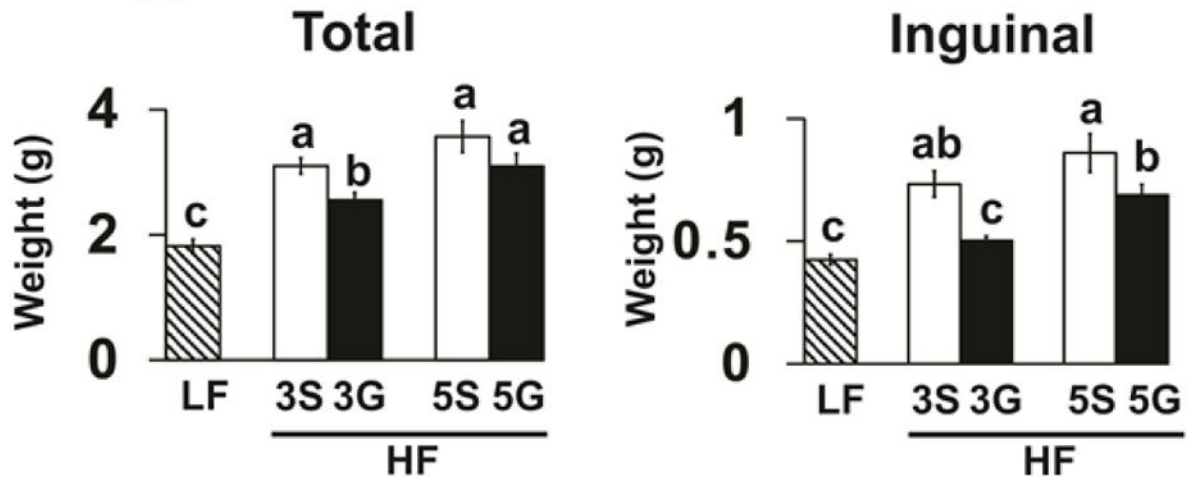


Figure 1.

Adiposity indices of C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Body fat percentages were measured at week 5 and week 10 using dual energy x-ray absorptiometry (DEXA). (B) At week 11, epididymal, inguinal, retroperitoneal, and mesenteric white adipose tissue (WAT) depots were excised and weighed. The weights of the epididymal, inguinal, retroperitoneal, and mesenteric depots were measured, and their sum labelled total WAT. Means \pm SEM without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's *t* test. Means \pm SEM ($n = 9-10$) sharing the symbol "*" differ using the Bonferroni's adjustment ($p < 0.01$). 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar; 5G, HF diet containing 5% grapes.

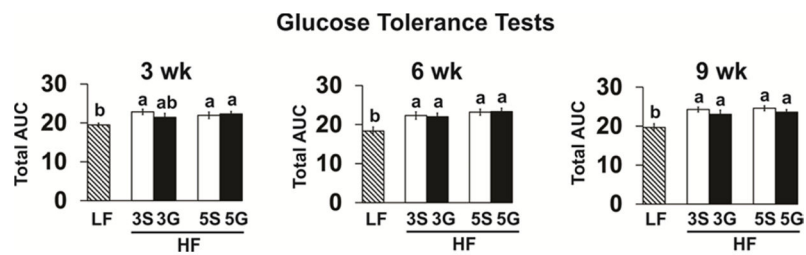
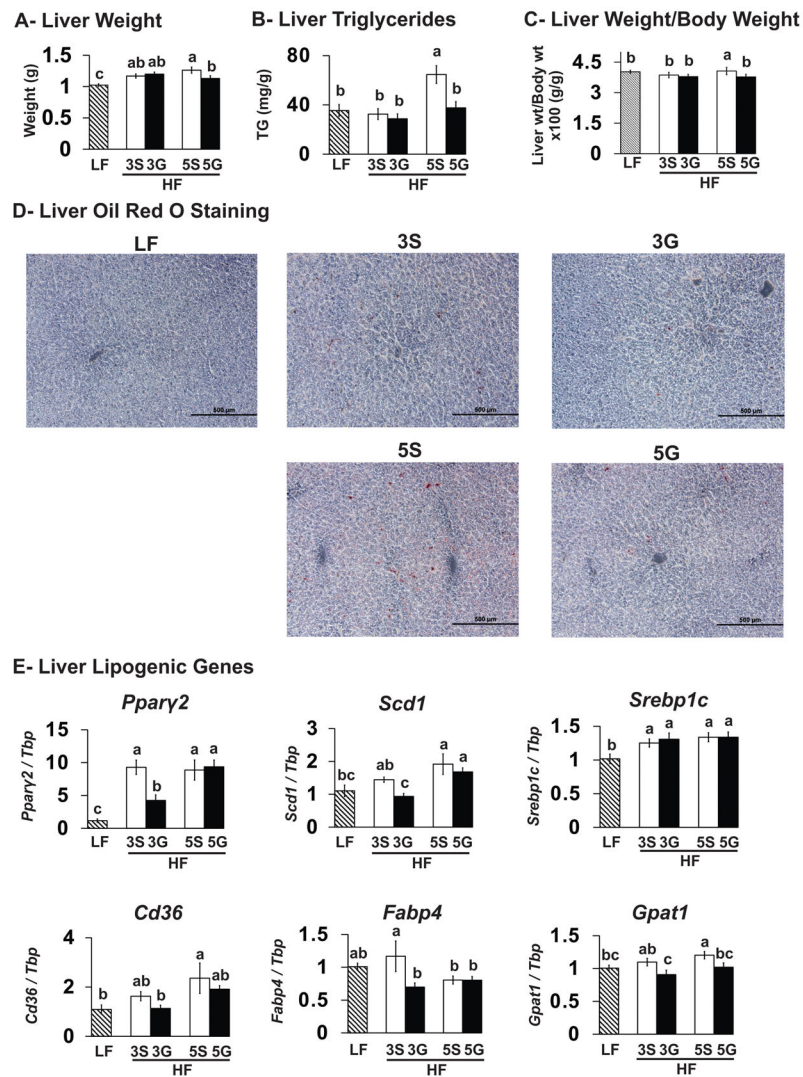


Figure 2.

Glucose tolerance tests (GTT)s of C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. At weeks 3, 6, and 9, GTTs were conducted on mice fasted for 8 h and injected i.p. with a 20% glucose solution. Data are expressed as total area under the curve (AUC) for the GTTs. Means \pm SEM (n=9–10) without a common lowercase letter differ (p<0.05) using one-way ANOVA and Student's t test. 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar; 5G, HF diet containing 5% grapes.

**Figure 3.**

Liver weights (A), liver triglyceride levels (B), ratio of liver weight to body weight (BW) (C), Oil red-O staining of liver, and the expression of several lipogenic genes in liver (E) of C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. qPCR was conducted to measure mRNA abundance of genes associated with hepatic lipogenesis. Means \pm SEM (n=9–10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test. 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar; 5G, HF diet containing 5% grapes.

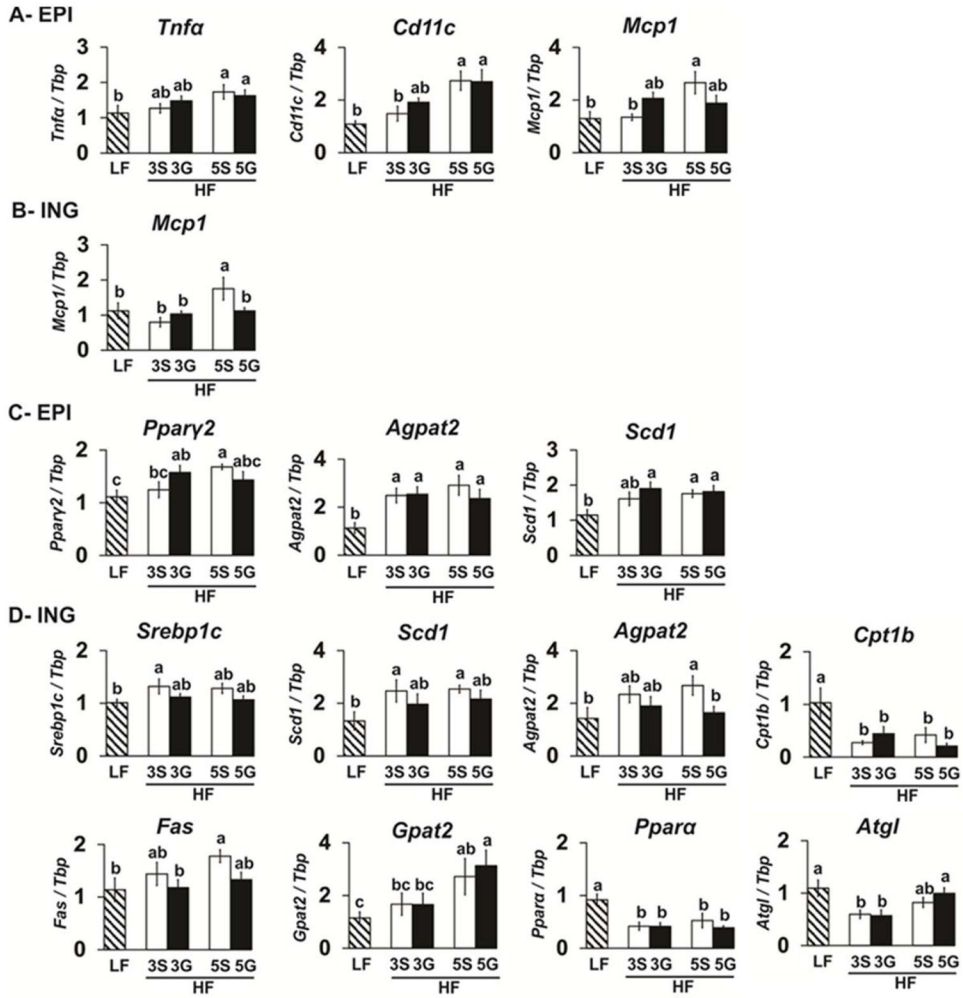


Figure 4. The expression of markers of inflammation and lipid metabolism in epididymal and inguinal WAT of C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. qPCR was conducted to measure mRNA abundance of genes associated with inflammation and lipid metabolism in epididymal (EPI; visceral) (A, C) and inguinal (ING; subcutaneous) (B, D) WAT depots. Means ± SEM (n=9–10) without a common lowercase letter differ (p<0.05) using one-way ANOVA and Student’s t test. 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar; 5G, HF diet containing 5% grapes.

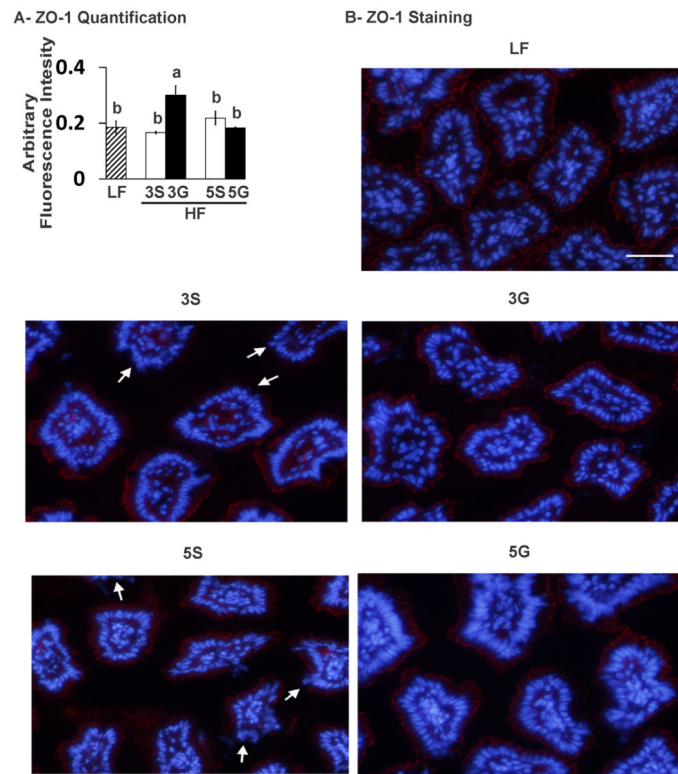
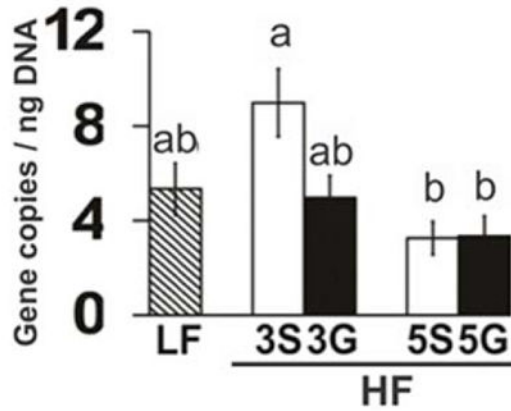


Figure 5.

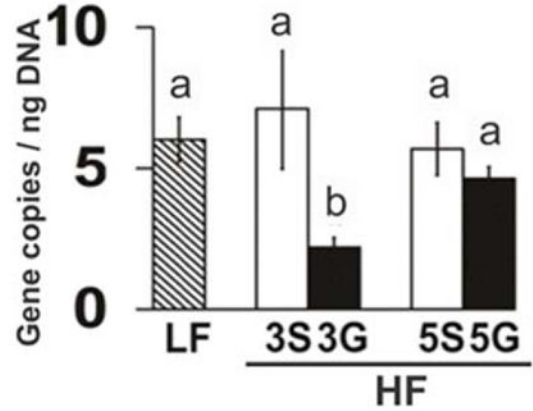
Ileal mucosa localization of the tight junction protein ZO-1 at the apical area of the ileum epithelium in C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. Localization of ZO-1 was visualized by immunostaining of ileum samples, and the staining was quantified (n=3–4). Arrows indicate impaired localization of ZO-1 at the apical area of the ileum epithelium. Means \pm SEM without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test. 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar; 5G, HF diet containing 5% grapes.

A-Sulfidogenic Bacteria

dsrA Bw

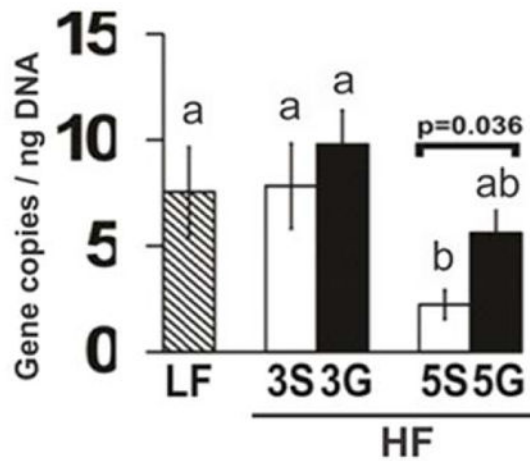


DSB



B-*Akkermansia muciniphila*

P. colon mucosa



Colon Digesta

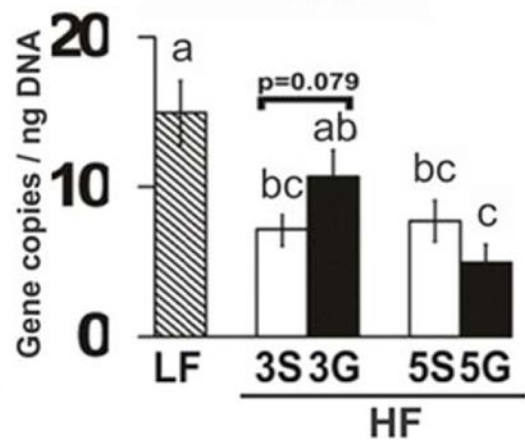


Figure 6.

Abundance of sulfidogenic bacteria and *A. muciniphila* in the intestinal mucosa or digesta of C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. Abundance of *B. wadsworthia*-specific, functional gene target dissimilatory sulfate reductase (*dsrA-Bw*) and the targeted sulfidogenic bacterial genus *Desulfobacter* (DSB) species in the ileum mucosa (A). Abundance of *A. muciniphila* in colonic mucosa and digesta and in cecal digesta (B). qPCR was conducted to measure abundance of 16S rRNA and functional genes. Means \pm SEM (n=9–10) without a common lowercase letter differ ($p < 0.05$) using one-way ANOVA and Student's t test. 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar;

5G, HF diet containing 5% grapes. Means \pm SEM (n=9–10) without a common lowercase letter differ ($p < 0.05$).

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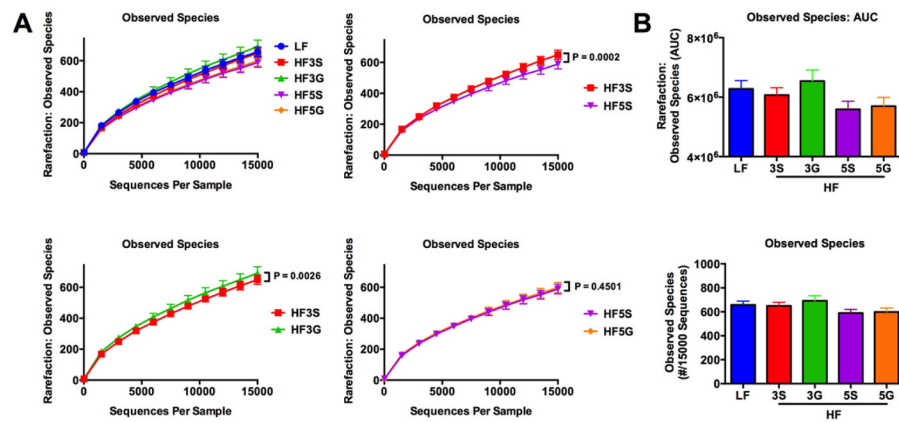


Figure 7.

Observed bacterial taxa in cecal mucosa of C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Rarefaction curves of observed species are shown. Samples were rarified to 15,000 sequencing reads per sample. (B) Above - Area Under the Curve (AUC) of rarefaction curves shown for each diet group. Below - Observed species at 15,000 sequencing reads for each diet group. 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar; 5G, HF diet containing 5% grapes. Data are presented as means \pm SEM (n=9–10) using one-way ANOVA and Dunn’s test for multiple comparisons.

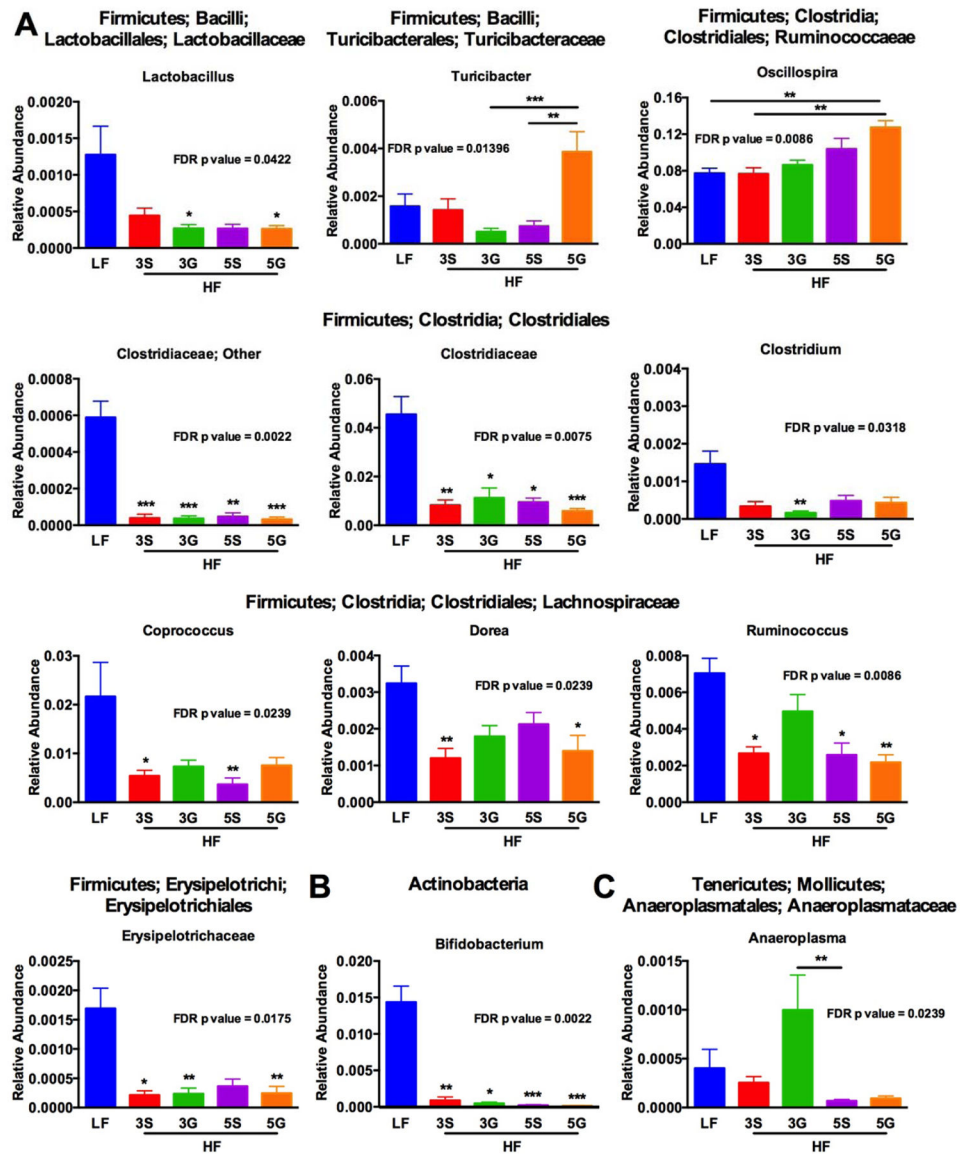


Figure 8. Significantly altered relative abundances of microbial taxa (i.e., A- Firmicutes, B- Actinobacteria, and C- Tenericutes) found across C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. Taxa shown were significantly altered based on Kruskal Wallis test run using QIIME software following filtering of OTUs that were not present in 50% of samples. FDR corrected p values based on this analysis are shown. To conduct multiple comparisons relative abundances were analyzed via ANOVA followed by Dunn's test for multiple comparisons. 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar; 5G, HF diet containing 5% grapes. Data are presented as means \pm SEM (n=9–10). Unless otherwise indicated, asterisks show significant differences of HF diets with or without grapes compared to LF control.

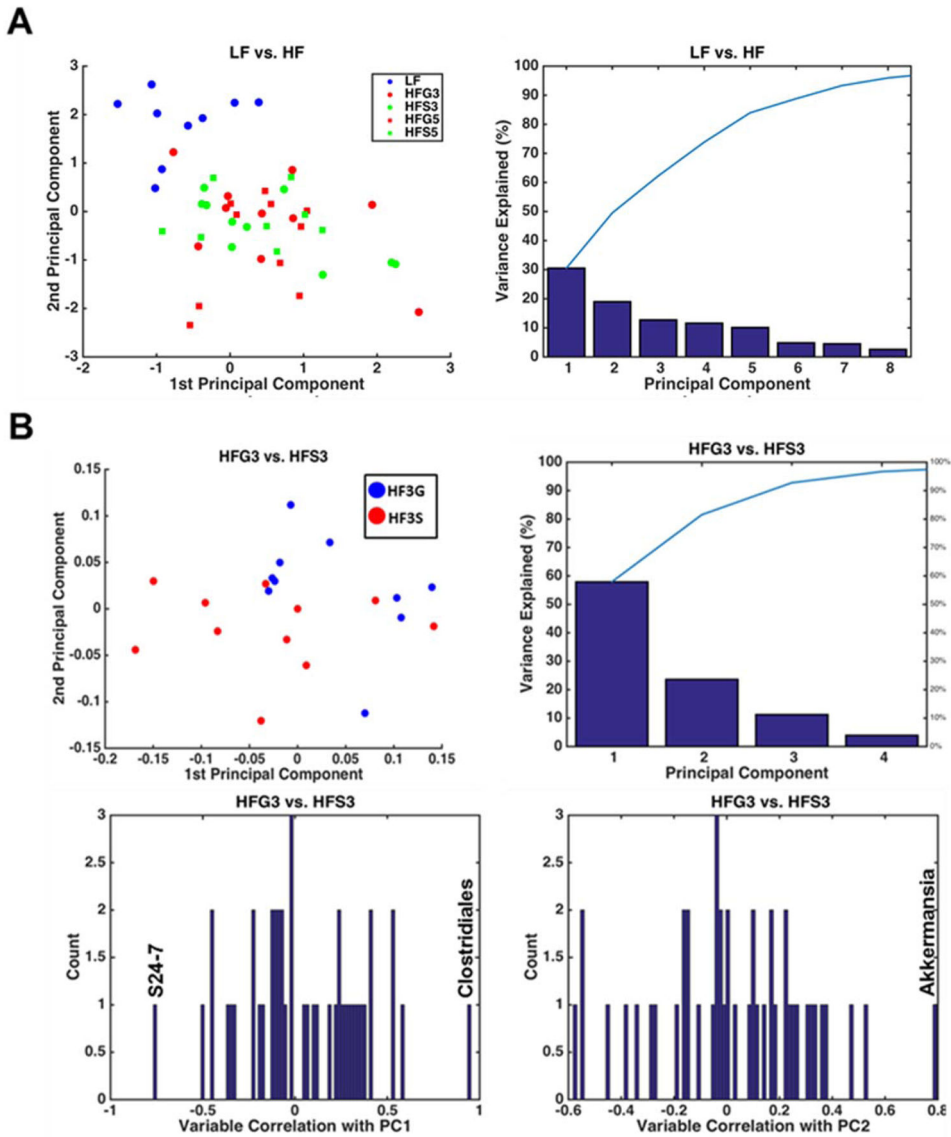


Figure 9. The microbial relative abundances from C57BL/6J mice fed a low fat (LF) diet or high fat (HF), butter-rich diets with or without 3% or 5% powdered grapes for 10 weeks. (A) Top Left - PCA plot between low fat (LF) and HF + 3% grapes (HF3G) are shown. Top Right – Pareto plot showing percentage of variance explained by principal components. Below – Histogram showing individual genera that were negatively or positively correlated with LF and/or HF-3G diets. (B) Same as A, but showing PCA of HF-3G vs HF + 3% sugar (HF-3S) groups.

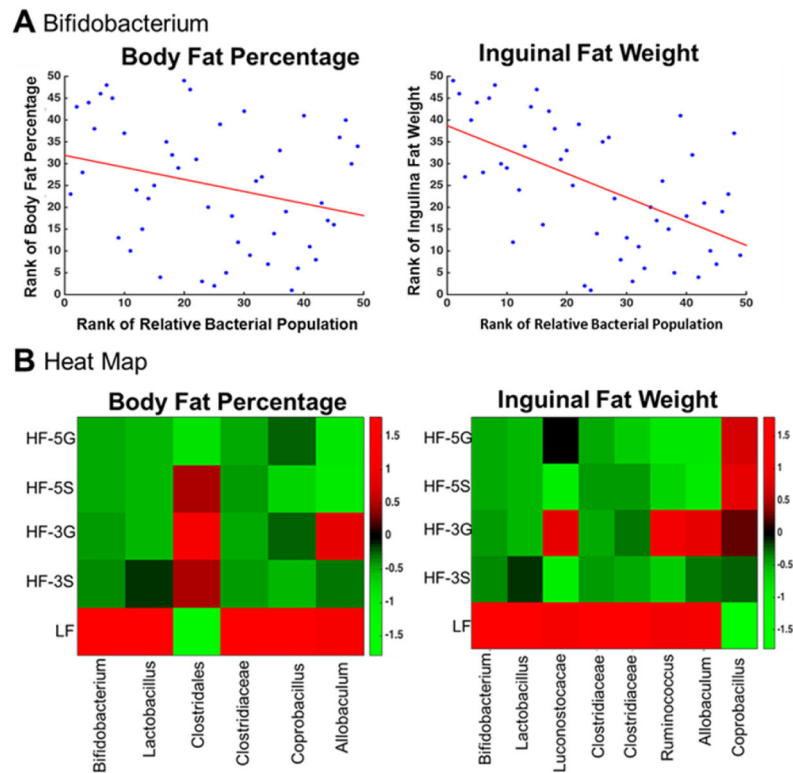


Figure 10.

(A) *Bifidobacterium* spp. relative abundance is negatively correlated with body fat percentage ($r = -0.53$, $p = 0.001$) and inguinal fat pad weight ($r = -0.48$, $p = 0.004$) as determined by Spearman Correlation Analysis. (B) Heatmap showing relative abundances of taxa that were significantly correlated with body fat percentage and inguinal fat pad weight. Increasing intensity of red color signifies higher abundance and increasing intensity of green color signifies lower relative abundance of taxa shown. LF, low fat; HF, high fat; 3S, HF diet containing 3% sugar; 3G, HF diet containing 3% grapes; 5S, HF diet containing 5% sugar; 5G, HF diet containing 5% grapes.

Table 1

The effect of diet composition on total body weight gain, total food intake, food conversion efficiency, and total caloric intake

Diets	BWG n=9-10	FI n=5	FCE n=5	Kcal n=5
LF	7.0 ± 0.6 ^b	399 ± 8 ^b	28.6 ± 1.2 ^a	1534 ± 26 ^b
HF-3G	13.1 ± 0.7 ^a	465 ± 7 ^a	17.8 ± 0.8 ^b	2051 ± 26 ^a
HF-3S	11.5 ± 0.7 ^a	424 ± 20 ^{ab}	18.7 ± 0.9 ^b	1874 ± 80 ^a
HF-5G	11.3 ± 1.1 ^a	409 ± 12 ^{ab}	18.4 ± 1.1 ^b	1799 ± 45 ^{ab}
HF-5S	12.6 ± 1.0 ^a	438 ± 25 ^{ab}	17.8 ± 1.5 ^b	1957 ± 124 ^a

Mean ± SEM without a common lower case letter in a column differ ($p < 0.05$) using one-way ANOVA and Student's t-test. BWG= total body weight gain (g). FI= total food intake per cage (g). FCE= food conversion efficiency per cage. Kcal= total caloric intake per cage (kcal).

Table 2

Statistical Analyses of Unifrac Distances

Weighted	R2	P Value
Adonis	0.12361	0.001
Anosim	0.3048	0.01
	Pseudo F Statistic	p value
Permanova	1.4105	0.001
Unweighted	R2	P Value
Adonis	0.23825	0.001
Anosim	0.2609	0.01
	Pseudo F Statistic	p value
Permanova	3.1277	0.001

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Table 3

Taxa Associated with Body Fat

Taxa Associated with Body Fat Percentage: (Phylum; Class; Order; Family; Genus; Species)	Correlation	P Value
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	-0.53	0.0001
Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	-0.33	0.0221
Firmicutes; Clostridia; Clostridiales; Other; Other	0.39	0.0063
Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Other	-0.32	0.0243
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus	-0.35	0.0147
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum	-0.30	0.0369
Taxa Associated with Inguinal Fat Pad Weight: (Phylum; Class; Order; Family; Genus; Species)	Correlation	P Value
Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium	-0.48	0.0004
Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	-0.39	0.0053
Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Leuconostoc	-0.29	0.0413
Firmicutes; Clostridia; Clostridiales; Other; Other	0.38	0.0065
Firmicutes; Clostridia; Clostridiales; Clostridiaceae	-0.34	0.0177
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Ruminococcus	-0.35	0.0131
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Allobaculum	-0.30	0.0391
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Coprobacillus	0.31	0.0297
Tenericutes; Mollicutes; Anaeroplasmatales; Anaeroplasmataceae; Anaeroplasma	-0.30	0.0380