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Influence of whole-wheat consumption on fecal microbial ecology of obese diabetic mice

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The digestive tract of mammals and other animals is colonized by trillions of metabolically active microorganisms. Changes in the gut microbiota have been associated with obesity in both humans and laboratory animals. Dietary modifications can often modulate the obese gut microbial ecosystem towards a more healthy state. This phenomenon should preferably be studied using dietary ingredients that are relevant to human nutrition. This study was designed to evaluate the influence of whole-wheat, a food ingredient with several beneficial properties, on gut microorganisms of obese diabetic mice. Diabetic (db/db) mice were fed standard (obese-control) or WW isocaloric diets (WW group) for eight weeks; non-obese mice were used as control (lean-control). High-throughput sequencing using the MiSeq platform coupled with freely-available computational tools and quantitative real-time PCR were used to analyze fecal bacterial 16S rRNA gene sequences. SCFAs were measured in feces using HPLC-PDA. Results showed no statistical difference in final body weights between the obese-control and the WW group. Almost 9,000 different bacterial species (Operational Taxonomic Units at 97% similarity) were detected in all mice but the bacterial diversity (number of OTUs) did not differ among the treatment groups. The abundance of Ruminococcaceae, a family containing several butyrate-producing bacteria, was found to be higher in obese (median: 6.9%) and WW-supplemented mice (5.6%) compared to lean (2.7%, $p = 0.02$, Kruskal-Wallis test). Fecal concentrations of butyrate were higher in obese (average: 2.91 mmol/mg of feces) but especially in WW-supplemented mice (4.27 mmol/mg) compared to lean controls (0.97 mmol/mg), while fecal succinic acid was lower in the WW group compared to obese but especially to the lean group. WW consumption was associated with ~3 times more relative abundance of *Lactobacillus* spp. compared to both obese and lean control mice. PCoA plots of weighted UniFrac distances revealed a distinctive clustering of lean microbial communities separately from both obese and WW-supplemented mice ($p = 0.001$, ANOSIM test). Predictive metagenome analysis using PICRUSt revealed significant differences in several metabolic features of the microbiota among the treatment groups, including fatty acid biosynthesis, carbohydrate and energy metabolism as well as synthesis and degradation of ketone bodies ($p < 0.01$). However, obese and WW groups tended to share more similar

abundances of gene families compared to lean mice. Using an *in vivo* model of obesity and diabetes, this study suggests that daily WW supplementation for eight weeks may not be enough to influence body weight or to output a lean-like microbiome, both taxonomically and metabolically. However, WW-supplementation was associated with several statistically significant differences in the gut microbiome compared to obese controls that deserve further investigation.

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Influence of whole-wheat consumption on fecal microbial ecology of obese diabetic mice

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Keywords

Fecal Microbiota; High-throughput sequencing; Metabolic Pathways; Obesity; Whole-Wheat.

Abstract

The digestive tract of mammals and other animals is colonized by trillions of metabolically active microorganisms. Changes in the gut microbiota have been associated with obesity in both humans and laboratory animals. Dietary modifications can often modulate the obese gut microbial ecosystem towards a more healthy state. This phenomenon should preferably be studied using dietary ingredients that are relevant to human nutrition. This study was designed to evaluate the influence of whole-wheat, a food ingredient with several beneficial properties, on gut microorganisms of obese diabetic mice.

Diabetic (db/db) mice were fed standard (obese-control) or WW isocaloric diets (WW group) for eight weeks; non-obese mice were used as control (lean-control). High-throughput sequencing using the MiSeq platform coupled with freely-available computational tools and quantitative real-time PCR were used to analyze fecal bacterial 16S rRNA gene sequences. SCFAs were measured in feces using HPLC-PDA.

Results showed no statistical difference in final body weights between the obese-control and the WW group. Almost 9,000 different bacterial species (Operational Taxonomic Units at 97% similarity) were detected in all mice but the bacterial diversity (number of OTUs) did not differ among the treatment groups. The abundance of Ruminococcaceae, a family containing several butyrate-producing bacteria, was found to be higher in obese (median: 6.9%) and WW-supplemented mice (5.6%) compared to lean (2.7%, $p = 0.02$, Kruskal-Wallis test). Fecal concentrations of butyrate were higher in obese (average: 2.91 mmol/mg of feces) but especially in WW-supplemented mice (4.27 mmol/mg) compared to lean controls (0.97 mmol/mg), while fecal succinic acid was lower in the WW group compared to obese but especially to the lean group. WW consumption was associated with ~3 times

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more relative abundance of *Lactobacillus* spp. compared to both obese and lean control mice. PCoA plots of weighted UniFrac distances revealed a distinctive clustering of lean microbial communities separately from both obese and WW-supplemented mice (p = 0.001, ANOSIM test). Predictive metagenome analysis using PICRUSt revealed significant differences in several metabolic features of the microbiota among the treatment groups, including fatty acid biosynthesis, carbohydrate and energy metabolism as well as synthesis and degradation of ketone bodies ($p < 0.01$). However, obese and WW groups tended to share more similar abundances of gene families compared to lean mice.

Using an *in vivo* model of obesity and diabetes, this study suggests that daily WW supplementation for eight weeks may not be enough to influence body weight or to output a lean-like microbiome, both taxonomically and metabolically. However, WW-supplementation was associated with several statistically significant differences in the gut microbiome compared to obese controls that deserve further investigation.

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INTRODUCTION

Obesity is an epidemic with catastrophic consequences for the health of millions of people around the globe. Different strategies can help reduce body weight including changes in exercise and dietary habits, yet many patients genuinely struggle to successfully decrease their body weight due to multiple interrelated factors (*Gupta, 2014*).

The mammalian digestive tract is a complex organ that has been constantly co-evolving with trillions of microorganisms (the gut microbiota) to combat environmental pathogens and maximize food digestion for at least 600 million years. Despite its general resilience, the gut microbiota is still susceptible to changes in dietary and other life habits, some of which can lead to imbalances and consequently to disease (*Lozupone et al., 2012*). For instance, substantial evidence has been published showing an association between obesity and changes in gut microbial populations and its metabolism of dietary and endogenous compounds (*Delzenne et al., 2011*). Interestingly, the differences in gut microbial communities between lean and obese individuals are not irreversible (*Turnbaugh et al., 2008*) with diet being the most practical alternative to reestablish microbial equilibrium within the gut. Understanding changes in gut microorganisms in response to dietary modifications is essential to develop effective dietary strategies to help obese patients.

Growing evidence shows that the consumption of specific dietary ingredients or supplements such as probiotics, prebiotics, polyphenols, as well as whole-grains has the potential of modifying gut health parameters in obese individuals, both in humans and animal models (*Katcher et al., 2008; Noratto et al., 2014; Petschow et al., 2013; Vitaglione*

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93 *et al., 2015*). Whole-wheat (WW) is often recommended by medical nutritionists as part of
94 a healthy diet for both overweighed and lean individuals. While several investigations have
95 previously addressed the nutritional benefits of consuming WW (*Stevenson et al., 2012*),
96 very few studies have researched the potential of either WW or its individual nutrients to
97 alter the gut microbiota of lean or obese individuals (*Neyrinck et al., 2011*) or as part of
98 dietary management to treat obesity. One study investigated the effect of replacing refined
99 wheat with whole-grain wheat for 12 weeks on body weight and fat mass in overweighed
100 women (*Kristensen et al., 2011*). This short-period of 12 weeks was enough to significantly
101 reduce percentage fat mass but no body weights. Here we show that an 8-week
102 consumption period of an isocaloric WW diet did not significantly change body weights in
103 obese-diabetic mice. Overall, obese mice under WW-supplemented diet showed similarities
104 to obese controls with regards to gut microbial composition and predicted metabolic
105 profile. The effect of WW was mostly observed on fecal concentrations of butyrate and
106 succinate and a few bacterial groups such as *Lactobacillus*. The results may have
107 implications in clinical dietary management of obesity using WW.
108
109 **METHODS**
110 **Study design**
111 The Institutional Animal Care Use Committee from Washington State University approved
112 all experimental procedures (animal protocol approval number: 04436-001). Two strains of
113 male mice were used in this study, BKS.Cg- + *Lepr^{db}/+Lepr^{db}/OlaHsd* obese diabetic
114 (db/db), and lean BKS.Cg-*Dock7m +/+ Leprdb/OlaHsd* (Harlan Laboratories, Kent, WA).
115 Animals were purchased at 5-6 weeks of age and maintained in ventilated rack system with

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116 food and water provided *ad libitum* throughout the study. We received 11 mice for the lean
117 group and 10 mice from all other groups. After 7 days of acclimatization, obese mice were
118 randomly divided into two groups (n=10 each) namely obese (AIN-93 G Purified Rodent
119 Diet) and WW (whole-wheat supplemented diet). The wild type mice group (n=11) was
120 named lean (AIN-93 Diet). Diets were made by Dyets Inc. (Bethlehem, PA) (Table 1). Four
121 or five mice per cage were housed in an environment-controlled room (23 °C, 12 hours
122 dark-light cycle). All mice were visually inspected every day and body weight was
123 recorded from all animals once a week.
124
125 **Fecal collection and DNA extraction**
126 Fresh distal colon contents (see qPCR analysis below) and fecal samples were obtained
127 from all mice at the end of the study (8 weeks) and stored at -80 ºC prior to DNA and 16S
128 rRNA gene profiling analysis. Total DNA was extracted from at least two different fecal
129 pellets weighting approximately 200 mg. Following bead-beating, the QIAamp DNA Stool
130 Mini Kit (Qiagen Inc., Valencia, CA) was used for DNA extraction following the
131 manufacturer's instructions. DNA concentration and purity was determined using a
132 NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to a
133 working concentration of 5 ng/µL.
134
135 **High-throughput sequencing of 16S rRNA genes**
136 Amplification and sequencing were performed as described elsewhere (*Bokulich et al.,*
137 *2014*). Briefly, the V4 semi-conserved region of bacterial 16S rRNA genes was amplified
138 using primers F515 (5′–GTGCCAGCMGCCGCGGTAA–3′) and R806 (5′–

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GGACTACHVGGGTWTCTAAT–3′), with the forward primer modified to contain a unique 8-nt barcode and a 2-nt linker sequence at the 5′ terminus. Amplicons were combined into two separated pooled samples and submitted to the University of California Davis Genome Center DNA Technologies Core for Illumina paired-end library preparation, cluster generation, and 250-bp paired-end sequencing on an Illumina MiSeq instrument in two separate runs. For data analysis, raw Illumina fastq files were demultiplexed, quality filtered, and analyzed using the freely available Quantitative Insights into Microbial Ecology (QIIME) Virtual Box v.1.8.0 (*Caporaso et al., 2010*). Operational taxonomic units (OTUs) were assigned using two different approaches: first, using UCLUST v.1.2.22 (*Edgar, 2010*) as implemented in QIIME using the open-reference clustering algorithm described in (*Rideout et al., 2014*) for alpha and beta diversity analyses; and second, using the pick_closed_reference_otus.py QIIME script for further analysis using PICRUSt (see Predicted metabolic profiles below). The Greengenes 13_5 97% OTU representative 16S rRNA gene sequences was used as the reference sequence collection (*DeSantis et al., 2006*). Alfa and beta diversity analyses were performed using 3000 random sequences per sample (lowest number of sequences in a sample after demultiplexing, filtering and OTU picking). Raw sequences were uploaded into the Sequence Read Archive at NCBI (accession number: PRJNA281761). The trim.seqs command in MOTHUR (*Schloss et al., 2009*) was used for splitting original fastq files per sample for uploading to SRA.

Predicted metabolic profiles

OTUs from the closed_reference script were normalized and used to predict metagenome functional content using the online Galaxy version of PICRUSt (Phylogenetic Investigation

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of Communities by Reconstruction of Unobserved States) (*Langille et al., 2013*). PICRUSt uses existing annotations of gene content as well as 16S copy numbers from reference microbial genomes in the IMG database (*Markowitz et al., 2012*) and a functional classification scheme to catalogue the predicted metagenome content. The current galaxy version supports three types of functional predictions; this current study used the popular KEGG Orthologs (*Kanehisa et al., 2012*). STAMP (*Parks et al., 2010*) was used to visualize and analyze the PICRUSt data.

Quantitative real-time PCR (qPCR) analysis

DNA was extracted from distal colon content using the ZR Fecal DNA MiniPrep™ kit following the manufacturer's protocol (Zymo Research, Irvin, CA). qPCR was used to detect specific bacterial groups as described elsewhere (*Noratto et al., 2014*). Table 2 shows the primers sequences used for all qPCR analyses.

Measurement of short chain fatty acids (SCFAs) in caecal contents

SCFAs were quantified as reported elsewhere (*Campos et al., 2012*). Briefly, samples were analyzed by an HPLC-PDA system using an Aminex HPX-87H strong cation-exchange resin column (300 x 7.8 mm) and fitted with an ion exchange microguard refill cartridge (Bio-Rad, Hercules, CA). The HPLC-PDA system consisted of a Water 2695 Separation Module (Waters, Milford, MA), which was equipped with a Water 2996 photodiode array detector (PDA). Samples (20 µL) were eluted isocratically with 5 mM sulfuric acid at 0.6 mL/min, and the column temperature was held at 50 ºC. Sodium butyrate, acetic acid,

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oxalic acid, and succinic acid were identified and quantified by comparing retention time and UV-Visible spectral data to standards.

Statistical analysis

ANOVA or the non-parametric alternative Kruskal-Wallis test were used to analyze SCFAs concentrations and relative abundance of microbial groups depending on sample size and normality of residuals. Multiple-comparisons were performed using Tukey and Mann-Whitney tests. The Bonferroni and False Discovery Rate corrections were used to adjust for multiple comparisons. Analysis of Similarities (ANOSIM) was used to test for clustering of microbiotas using weighted and unweighted UniFrac distance matrices. QIIME v.1.8.0, R v.3.0.3 (*R core team*) and Excel were used for statistics and graphics. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to assess differences in microbial communities using taxa with at least 20 non-zero values and a LDA score threshold of >3.5 (*Segata et al., 2011*). Unless otherwise noted, an alpha of 0.05 was considered to reject null hypothesis.

RESULTS

One mouse in the obese group died for reasons unrelated to this study. At the end of the study, there was a significant ($p < 0.01$) difference in body weight between the lean (average: 30.6±2.2 g) and both the obese (46.1±2.8 g) and WW groups (45.3±5.8 g). WW consumption was not associated with a lower body weight compared to obese control group ($p = 0.96$, ANOVA).

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Fecal microbiota composition

A total of 8686 different OTUs were detected using the open reference algorithm described by *Rideout et al. (2014).* On the other hand, the closed_reference method used to generate data for PICRUSt (see PICRUSt below) only yielded 1302 OTUs. Fecal microbial composition of all mice was mostly comprised by Firmicutes (average: 58.7% across all samples) and Bacteroidetes (average: 32.8%) (Fig. 1). Other less abundant Phyla were Actinobacteria (~4%), Proteobacteria (~3%) and Verrucomicrobia (~0.8%) (Fig. 1). At the phylum level, lean mice had more Firmicutes (median: 64.9%) compared to both obese and WW (52.2% and 53.2% respectively) but this difference did not reach statistical significance ($p = 0.42$, Kruskal-Wallis test), partly because of the high variability among individual mice. The phylum Bacteroidetes was higher in the obese (median: 44.6%) and the WW group (median: 40.4%) compared to the lean group (median: 25.1%) but only the comparison between WW and lean reached statistical significance ($p = 0.02$, Bonferroni-corrected Mann-Whitney test). The ratio Bacteroidetes/Firmicutes was lower in the lean (median: 38.7%) compared to the obese group (median: 85.3%) and the WW group (median: 75.4%) but this difference did not reach significance ($p = 0.12$, Kruskal-Wallis). Two low abundant phyla (Actinobacteria and Verrucomicrobia) were higher in lean compared to both obese and WW groups ($p < 0.01$, Kruskal Wallis), while Proteobacteria was lower in lean (2.2%) and obese (1.9%) compared to WW group (4.4%) although this difference did not reach significance ($p = 0.13$, Kruskal-Wallis test) (Fig. 1).

Other differences among the treatment groups were also found at several taxonomic levels within the main phyla, an observation that was confirmed using LEfSe (Fig. 2). Overall,

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there were more similarities between obese and WW groups compared to lean although WW-supplementation seemed to output a unique pattern of bacterial abundances (Fig. 1). Among the bacterial groups that showed differences indicating an effect of WW-supplementation include the genus *Lactobacillus*, which was higher in WW-supplemented group (median: 26.1%) compared to lean and obese (medians: 9.7% and 6.9% respectively) ($p = 0.02$, Kruskal-Wallis test). The obese group had higher *Turicibacter* (3.4%) compared to the lean and the WW group (median: 0%) ($p = 0.01$, Kruskal-Wallis test) and this was confirmed using qPCR (see qPCR below). Within the Bacteroidetes, the controversial S24-7 family (see Thread in QIIME google group in references) was found to be higher in the WW group (median: 31.6%) compared to lean (median: 12.1%) and obese (median: 18.8%, $p = 0.003$, Kruskal-Wallis test).

Other differences in bacterial abundances suggested that WW-supplementation did not generate a lean-like microbiome. For example, *Bifidobacterium* was the most abundant genus within the Actinobacteria and was higher in the lean group (median 5.9%) compared to both obese (median: 0.6%, $p = 0.002$) and WW groups (median: 0.9%, $p = 0.0005$ Kruskal-Wallis test). Also, and despite the differences in the phylum Proteobacteria, the family Enterobacteriaceae was found to be lower in lean (median: 0.2%) compared to both obese (median: 0.8%, $p = 0.0006$) and WW groups (median: 1.4%, $p = 0.0004$) (Bonferroni corrected, Mann-Whitney test) and this was confirmed using qPCR. Also the genus *Akkermansia* (phylum Verrucomicrobia) was higher in lean (median: 0.7%) compared to both the obese (median: 0%) and WW (median: 0%) groups ($p = 0.005$ for both comparisons, Bonferroni corrected, Mann-Whitney test), a finding that was also confirmed

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253 with qPCR. Other interesting differences include the family Ruminococcaceae that was
254 higher in the obese group (median: 6.9%) and more similar to the WW group (median:
255 5.6%) compared to lean (median: 2.7%, *p=0.02* Kruskal-Wallis test). Also, the genus
256 *Allobaculum* (class Erysipelotrichi) was found to be much higher in lean (median: 41.9%;
257 min: 14.9%; max: 60.2%) compared to both obese and WW groups (median: 0.1%; min:
258 0.1%; max: 0.3%, $p < 0.0001$ Kruskal-Wallis test). These multiple differences in relative
259 abundance of sequence reads were visualized by plotting a heatmap at the lowest
260 taxonomic level obtained from the open reference algorithm (*Rideout et al., 2014*) in
261 QIIME (Fig. 3), revealing a clear separation of lean individuals from both obese and WW-
262 supplemented mice. This plot also revealed that WW-supplementation output a unique
263 pattern of bacterial abundances (Fig. 3).
264
265 **Alpha diversity**
266 There was no significant difference in number of species (OTUs at 97% similarity) and
267 Chao1 diversity index (data not shown). Rarefied plots of number of OTUs showed that
268 more than the 3000 sequences per sample used in this study are needed to fully describe the
269 fecal microbiota of all mice (Fig. 4).
270
271 **Beta-diversity**
272 Principal Coordinate Analysis (PCoA) of weighted and unweighted UniFrac metrics
273 showed different clustering of microbial communities. Weighted (which takes phylogenetic
274 information as well as sequence abundance into account) metrics clearly showed a different
275 microbial structure in lean individuals compared to obese and WW groups (ANOSIM p =

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0.001) (Fig. 5). This was expected based on the clustering of lean subjects using relative abundance of sequence reads (Fig. 2). On the other hand, the qualitative (does not take sequence abundance into account) unweighted UniFrac analysis shows that the microbiota of the WW group clustered separately from the lean and obese groups (ANOSIM p = 0.001) (Fig. 5).

Predicted metabolic profile

The taxa predicted by 16S RNA marker gene sequencing was used to predict the functional profile of the fecal microbiome in all three experimental groups. Predicted genes related to fatty acid biosynthesis were lower in the lean group (average: 0.34±0.09%) compared to both obese (average: 0.43±0.02%) and WW (average: 0.44±0.04) groups ($p < 0.05$). Also, mice in the WW group had the lowest proportion of genes related to synthesis and degradation of ketone bodies ($p < 0.05$, ANOVA). Table 3 shows other metabolic features that showed statistically significant differences among the groups. Overall, obese and WW groups tended to share more similar abundances of gene families compared to lean mice, an observation that supports the differences in bacterial abundances.

qPCR assessment of microbiota in distal colon contents

We performed qPCR analysis for bacterial groups of interest to health in distal colon contents. Similarly to the sequencing results from fecal samples, qPCR results revealed several differences in relative abundance for different bacterial groups (Fig. 6).

SCFAs caecal concentrations

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There was a statistically significant difference among the treatment groups for several SCFAs in feces (Table 4). Butyrate concentrations were higher in the WW group compared to both the lean and the obese group ($p < 0.001$, Kruskal-Wallis). Also, WW consumption was associated with lower fecal succinic acid concentrations ($p = 0.009$, Kruskal-Wallis).

DISCUSSION

Obesity is a worldwide epidemic disease that has been associated with changes in the gut microbiome in many different studies. Consumption of whole grains is often recommended by medical nutritionists as part of a healthy diet. To our knowledge, this is the first study evaluating the *in vivo* effect of WW consumption on fecal microbial ecology of obese diabetic mice, adding valuable information to the literature with regard to the use and development of dietary strategies to help obese patients.

Ley et al. (2005) showed that lean mice have more Bacteroidetes and less Firmicutes compared to obese mice, a finding that has been reported by several other research groups. However, it is important to note that these observations were division-wide (in other words, there was no specific subgroup such as families or genera that were present high or low in abundance) and, more importantly, that other researchers have found either no difference in Firmicutes and Bacteroidetes between obese and lean (*Duncan et al., 2008*) or more Bacteroidetes in obese compared to normal-weight individuals (*Zhang et al., 2009*). Interestingly, in this study sequencing showed no statistical difference in the abundance of both phyla Firmicutes and Bacteroidetes between lean and obese control; nonetheless, two important aspects must be taken into account. First, obese and WW mice were consistently

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more like each other compared to lean mice with regard to the abundance of both phyla. Also, contrary to the observations by *Ley et al*. (2005), lean mice had more Firmicutes and less Bacteroidetes compared to both obese and WW mice, a difference that did not reach statistical significance. qPCR confirmed the results about Firmicutes but a discrepancy was observed with regard to Bacteroidetes, maybe due to the use of fecal (sequencing) or colon (qPCR) contents for bacterial analysis. Regardless, differences in taxa abundance at the phylum level have little relevance when considering all their individual groups within. For instance, many bacterial groups deserve attention, like the mucin-degrader *Akkermansia* which has been shown to be inversely correlated with body weight in rodents and humans (*Everard et al., 2013*). Accordingly, both sequencing and qPCR in this current study showed that obese mice had fewer *Akkermansia* and WW consumption surprisingly helped to decrease its abundance even further. Here it is important to note that a higher abundance in feces does not necessarily imply a higher abundance in the mucus. WW consumption was also associated with much more *Lactobacillus* spp., a bacterial genus frequently used in probiotic formulations, and the genus *Allobaculum* was practically absent in both obese and WW groups while lean individuals were heavily colonized by this group. These changes in bacterial abundances deserve more investigation.

Beta diversity metrics are useful to study similarities of microbiomes, which in turn have critical consequences for understanding health and disease processes. *Lozupone et al.* (2007) explains that quantitative beta-diversity measures (weighted UniFrac distances) are better for revealing community differences that are due to changes in relative taxon (OTUs) abundance, while qualitative (unweighted) are most informative when communities differ

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by what can live in them. Most studies report either weighted or unweighted but few report both. In this study, weighted analysis showed a clear separation of lean samples from all samples from the obese and WW groups, suggesting that the numbers of OTUs are an important determinant to separate lean microbiomes from obese individuals with and without WW. In this study, the results of weighted analysis also show that animal genetics was the predominant factor to separate microbiotas. On the other hand, unweighted analysis showed opposite results: lean and obese samples clustered separately from all samples of the WW group, suggesting that WW helped create an environment that favored a phylogenetically different ecosystem. At this point, both methods should be considered for explaining the changes in gut microbiomes in investigations like this study (*Lozupone et al. 2007*). In order to explain the discrepancy between the results of weighted and unweighted results, it is feasible to hypothesize that an 8-week period of WW consumption helped change the overall environment in the intestinal lumen, thus modulating what can live and proliferate in it (unweighted results). In turn, this different environment may have promoted changes in the abundance of specific taxa (weighted results), as shown in this study for several bacterial groups. Given that the assessment of microbial diversity is a major component in microbial ecological studies, and that these assessments closely relate to our understanding of health and health deviations, we expect others to start inspecting and reporting both weighted and unweighted UniFrac distance metrics. The use of both metrics has been shown to be useful in various investigations (*Campbell et al., 2015; Igarashi et al., 2014; Wu et al., 2010*).

Microbial butyrate is essential for colon health and lower concentrations of this fatty acid

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368 are usually considered non-optimal for gut health (*Donohoe et al., 2011*). Nonetheless,
369 studies have shown that obese individuals actually have higher fecal butyrate and other
370 SCFAs compared to lean individuals (*Fernandes et al., 2014*), an observation that suggests
371 that both lower and higher butyrate concentrations than normal may be associated with and
372 perhaps aggravate disease. Similarly, obese mice in this current study (with and without
373 WW supplementation) had higher fecal butyrate compared to lean mice. Butyrate-
374 producers are abundant in the mammalian gut and mainly belong to the family
375 Ruminococcaceae within the Firmicutes (*Louis et al., 2009*). In this study both sequencing
376 and qPCR revealed higher Ruminococcaceae in obese and WW groups compared to lean
377 individuals, thus potentially explaining the higher fecal butyrate concentrations. Another
378 SCFA that deserves attention is succinic acid, which has been shown to increase in rats fed
379 a high-fat diet (*Jakobsdottir et al., 2013*). In this current study, obese mice had lower
380 concentrations of succinic acid and WW-supplementation seemingly helped to drastically
381 decrease it. Unfortunately, far more attention has been paid to butyrate compared to
382 succinate, propionate and other SCFA (*Cheng et al., 2013; Reichardt et al., 2014*).
383
384 The assessment of microbial metabolic activity in complex ecosystems is hampered in part
385 by the huge number of microorganisms and the cost of sequencing either whole genomes or
386 transcriptomes. PICRUSt allows a prediction of the metabolic profile using taxa predicted
387 by 16S rRNA gene sequencing. PICRUSt is, however, not exempt of pitfalls: it only uses
388 information for well-defined 16S sequences and the presence of a given set of genes does
389 not tell anything about their functional activity depending on the specific environmental
390 conditions. Supported by the similarities in abundance of most bacterial groups between

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obese-control and WW groups, this study showed that 8-week WW consumption was not enough to make a significant difference in the abundance of bacterial gene families.

SUMMARY

In summary, this study suggests that an 8-week consumption of whole-wheat may not be enough to exert an effect on body weight and to output a lean-like microbiome using an *in vivo* model of obesity and diabetes. However, WW-supplementation was associated with several statistically significant changes that deserve further investigations. These results may or may not apply to obesity in human patients. The clinical relevance of this present work remains to be determined.

Caveats

This study was designed to obtain preliminary information about the influence of WW consumption on gut microbial ecology of obese diabetic mice; therefore, we did not aim to determine the exact compound(s) behind the observed effects. Wheat is a fiber-rich grain and consumption of fiber alone is associated with changes in the gut microbiome and the immune system of the host (*Bermudez-Brito et al., 2015*). Aside fiber, WW also contains other bioactive compounds (e.g. polyphenols) that may be responsible for specific effects on host metabolism, physiology and immune system. For instance, it has been recently shown that wheat-derived alkylresorcinols were capable of showing beneficial effects on diet-induced obese mice (*Oishi et al., 2015*). Interestingly, our group showed that carbohydrate-free polyphenol-rich juice from plum is capable of impeding body weight gain in obese Zucker rats (*Noratto et al., 2014*), a finding that was not observed with WW

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consumption in mice in this current study. More research is necessary to investigate the separate effect of the different nutrients in WW.

Future directions

In humans, obesity is a multifactorial disease that can be partly controlled with dietary modifications. This paper adds valuable information to the current literature with regard to the potential influence of WW consumption on the gut microbiota of obese diabetic mice. However, research is needed to investigate the effect of WW on obese human individuals.

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643 **TABLES**

644 **Table 1.** Formulation of experimental diets (g/100 g).

Ingredients	Lean and Obese diet	Wheat diet
Casein, high nitrogen	20	0.0
L-Cysteine	0.3	0.3
Whole-wheat meal	0.0	87.94
Soybean oil	7.0	7.0
Sucrose	10	0.0
Cornstarch	39.74	0.0
Dyetrose	13.2	0.0
t-Butylhydroquinone	0.0014	0.0014
Cellulose	5	0.0
Mineral mix #210025	3.5	3.5
Vitamin mix #310025	1.0	1.0
Choline bitartrate	0.25	0.25
Kcal/100g	376.00	387.76

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659 **Table 2**. Oligonucleotides used in this study for qPCR analyses.

qPCR primers	Sequence (5`-3`)*	Target	Reference
HDA1	ACTCCTACGGGAGGCAGCAGT	All bacteria (V2-V3 regions, position 339-539 in the *E. coli* 16S gene)	*Walter et al. 2000*
HDA2	GTATTACCGCGGCTGCTGGCAC		
Bact834F	GGARCATGTGGTTTAATTCGATGAT	Bacteroidetes (Phylum)	*Guo et al. 2008*
Bact1060R	AGCTGACGACAACCATGCAG		
928F-Firm	TGAAACTYAAAGGAATTGACG	Firmicutes (Phylum)	*Bacchetti et al. 2011*
1040firmR	ACCATGCACCACCTGTC		
BifF	GCGTGCTTAACACATGCAAGTC	*Bifidobacterium* (genus)	*Penders et al. 2005*
BifR	CACCCGTTTCCAGGAGCTATT		
E. coli F	CATGCCGCGTGTATGAAGAA	*E. coli*	*Huijsdens et al. 2002*
E. coli R	CGGGTAACGTCAATGAGCAAA		
TuriciF	CAGACGGGGACAACGATTGGA	*Turibacter* (genus)	*Suchodolski et al. 2012*
TuriciR	TACGCATCGTCGCCTTGGTA		
RumiF	ACTGAGAGGTTGAACGGCCA	Ruminococcaceae (family)	*Garcia-Mazcorro et al. 2012*
RumiR	CCTTTACACCCAGTAAWTCCGGA		
FaecaliF	GAAGGCGGCCTACTGGGCAC	*Faecalibacterium* (genus)	*Garcia-Mazcorro et al. 2012*
FaecaliR	GTGCAGGCGAGTTGCAGCCT		
Eco1457-F	CATTGACGTTACCCGCAGAAGAAGC	Enterobacteriaceae (family)	*Bartosch et al. 2004*
Eco1652-R	CTCTACGAGACTCAAGCTTGC		
V1F	CAGCACGTGAAGGTGGGGAC	*Akkermansia muciniphila*	*Collado et al. 2007*
V1R	CCTTGCGGTTGGCTTCAGAT		
PrevF	CACCAAGGCGACGATCA	*Prevotella* (genus)	*Larsen et al. 2010*
PrevR	GGATAACGCCYGGACCT		
Bfr-F	CTGAACCAGCCAAGTAGCG	*Bacteroides fragilis*	*Liu et al. 2003*
Bfr-R	CCGCAAACTTTCACAACTGACTTA		

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661 *The oligonucleotide sequences in this column used the IUPAC nucleotide codes.

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670 **Table 3**. Metabolic features in the Lean (n=11), Obese (n=9) and Whole-Wheat (n=10)
671 groups. Data was organized based on KEGG categories. This table only shows those
672 features that were significantly different among the treatment groups (ANOVA with *p*-
673 values adjusted for False Discovery Rate in STAMP).
674

KEGG gene categories			Treatment Groups			
			Lean	Obese	Whole-Wheat	
Level_1	Level_2	Level_3	Mean ± st. dev.	Mean ± st. dev.	Mean ± st. dev.	SS
Cellular Processes	Cell Motility	Bacterial chemotaxis	0.24±0.07	0.49±0.15	0.36±0.16	*
Cellular Processes	Cell Motility	Flagellar assembly	0.14±0.08	0.50±0.17	0.42±0.26	*
Cellular Processes	Transport and Catabolism	Peroxisome	0.15±0.03	0.18±0.04	0.23±0.03	**
Environmental Information Processing	Membrane Transport	Transporters	6.83±0.84	7.53±1.02	5.79±1.09	*
Environmental Information Processing	Membrane Transport	Phosphotransferase system (PTS)	0.98±0.40	0.51±0.23	0.45±0.22	*
Environmental Information Processing	Signaling Molecules and Interaction	Bacterial toxins	0.14±0.02	0.11±0.02	0.09±0.04	*
Environmental Information Processing	Signal Transduction	Phosphatidylinositol signaling system	0.11±0.01	0.08±0.01	0.10±0.01	**
Environmental Information Processing	Signal Transduction	MAPK signaling pathway - yeast	0.04±0.01	0.06±0.01	0.06±0.02	*
Environmental Information Processing	Signaling Molecules and Interaction	Ion channels	0.05±0.01	0.02 ±0.01	0.02 ±0.01	***
Genetic Information Processing	Folding, Sorting and Degradation	Chaperones and folding catalysts	0.98±0.05	0.99±0.08	1.10±0.08	*
Genetic Information Processing	Replication and Repair	Base excision repair	0.50±0.06	0.41±0.03	0.41±0.04	**
Human Diseases	Infectious Diseases	Tuberculosis	0.18±0.02	0.13±0.01	0.13±0.02	**
Human Diseases	Cancers	Pathways in cancer	0.07±0.01	0.04±0.00	0.05±0.00	***
Human Diseases	Neurodegenerative Diseases	Alzheimer's disease	0.06±0.01	0.05±0.00	0.05±0.01	*
Human Diseases	Neurodegenerative Diseases	Amyotrophic lateral sclerosis (ALS)	0.02±0.01	0.03±0.01	0.04±0.01	***
Human Diseases	Cancers	Renal cell carcinoma	0.03±0.01	0.01±0.00	0.01±0.01	***
Human Diseases	Neurodegenerative Diseases	Prion diseases	0.00±0.00	0.01±0.00	0.01±0.01	*
Metabolism	Carbohydrate Metabolism	Amino sugar and nucleotide sugar metabolism	1.74±0.30	1.44±0.09	1.41±0.18	*
Metabolism	Energy Metabolism	Methane metabolism	1.10±0.11	1.28±0.08	1.24±0.14	*
Metabolism	Carbohydrate Metabolism	Fructose and mannose metabolism	1.16±0.19	0.94±0.08	0.86±0.12	**
Metabolism	Energy Metabolism	Carbon fixation pathways in prokaryotes	0.90±0.09	0.94±0.10	1.07±0.11	*

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Metabolism	Carbohydrate Metabolism	Starch and sucrose metabolism	0.85±0.08	1.03±0.07	0.92±0.13	*
Metabolism	Carbohydrate Metabolism	Pentose phosphate pathway	0.92±0.05	0.86±0.07	0.78±0.06	**
Metabolism	Energy Metabolism	Nitrogen metabolism	0.66±0.03	0.70±0.06	0.76±0.08	*
Metabolism	Metabolism of Cofactors and Vitamins	Porphyrin and chlorophyll metabolism	0.55±0.15	0.85±0.13	0.66±0.14	**
Metabolism	Lipid Metabolism	Lipid biosynthesis proteins	0.46±0.12	0.55±0.02	0.59±0.04	*
Metabolism	Lipid Metabolism	Fatty acid biosynthesis	0.34±0.09	0.43±0.02	0.44±0.04	*
Metabolism	Metabolism of Other Amino Acids	Selenocompound metabolism	0.40±0.02	0.37±0.01	0.35±0.01	***
Metabolism	Xenobiotics Biodegradation and Metabolism	Drug metabolism - other enzymes	0.27±0.04	0.34±0.03	0.32±0.06	*
Metabolism	Metabolism of Other Amino Acids	Cyanoamino acid metabolism	0.23±0.04	0.33±0.02	0.30±0.06	**
Metabolism	Carbohydrate Metabolism	C5-Branched dibasic acid metabolism	0.23±0.06	0.32±0.02	0.31±0.02	**
Metabolism	Metabolism of Cofactors and Vitamins	Riboflavin metabolism	0.27±0.02	0.21±0.03	0.25±0.04	*
Metabolism	Metabolism of Other Amino Acids	beta-Alanine metabolism	0.18±0.04	0.22±0.04	0.28±0.06	**
Metabolism	Metabolism of Cofactors and Vitamins	Vitamin B6 metabolism	0.17±0.02	0.20±0.02	0.22±0.01	***
Metabolism	Biosynthesis of Other Secondary Metabolites	Phenylpropanoid biosynthesis	0.12±0.03	0.20±0.02	0.17±0.05	**
Metabolism	Metabolism of Cofactors and Vitamins	Biotin metabolism	0.13±0.03	0.14±0.03	0.18±0.02	*
Metabolism	Metabolism of Terpenoids and Polyketides	Biosynthesis of ansamycins	0.14±0.04	0.11±0.02	0.09±0.02	**
Metabolism	Xenobiotics Biodegradation and Metabolism	Polycyclic aromatic hydrocarbon degradation	0.14±0.03	0.10±0.01	0.10±0.03	*
Metabolism	Metabolism of Cofactors and Vitamins	Lipoic acid metabolism	0.04±0.01	0.05±0.02	0.07±0.01	*
Metabolism	Xenobiotics Biodegradation and Metabolism	Ethylbenzene degradation	0.03±0.01	0.05±0.02	0.06±0.02	*
Metabolism	Lipid Metabolism	Synthesis and degradation of ketone bodies	0.06±0.02	0.04±0.01	0.03±0.01	*
Metabolism	Biosynthesis of Other Secondary Metabolites	Flavonoid biosynthesis	0.02±0.01	0.01±0.00	0.00±0.00	***
Metabolism	Metabolism of Other Amino Acids	D-Arginine and D-ornithine metabolism	0.02±0.01	0.01±0.00	0.00±0.00	*
Metabolism	Biosynthesis of Other Secondary Metabolites	Stilbenoid, diarylheptanoid and gingerol biosynthesis	0.01±0.01	0.00±0.00	0.00±0.00	**
Organismal Systems	Environmental Adaptation	Plant-pathogen interaction	0.14±0.01	0.16±0.02	0.13±0.01	*
Organismal Systems	Digestive System	Mineral absorption	0.00±0.00	0.00±0.00	0.00±0.00	*

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678 **Table 4.** Median (minimum-maximum) for all short-chain fatty acids (SCFAs). Results are
679 expressed in mmol/mg of feces.

SCFA	Lean	Obese	Whole-wheat	*p* value
Sodium butyrate	0.97 (0.15-2.65)	2.91 (1.47-4.35)†	4.27 (3.05-6.26)†‡	<0.001
Acetic acid	10.2 (7.7-26.3)	12.0 (8.3-18.7)	15.4 (10.1-31.9)	0.208
Oxalic acid	15.14(6.68-18.91)	14.60 (8.78-28.01)	9.96 (6.76-12.15)†	0.033
Succinic acid	39.84 (15.29-97.63)	22.97 (3.86-71.18)	3.12 (0.91-63.36)†‡	0.009

680

681 † Significantly different compared to lean group ($p < 0.05$). ‡ Significantly different
682 compared to obese group ($p < 0.05$). *p* values come from the Kruskal-Wallis test and
683 multiple comparisons were performed using the Mann-Whitney test and corrected with the
684 Bonferroni method.

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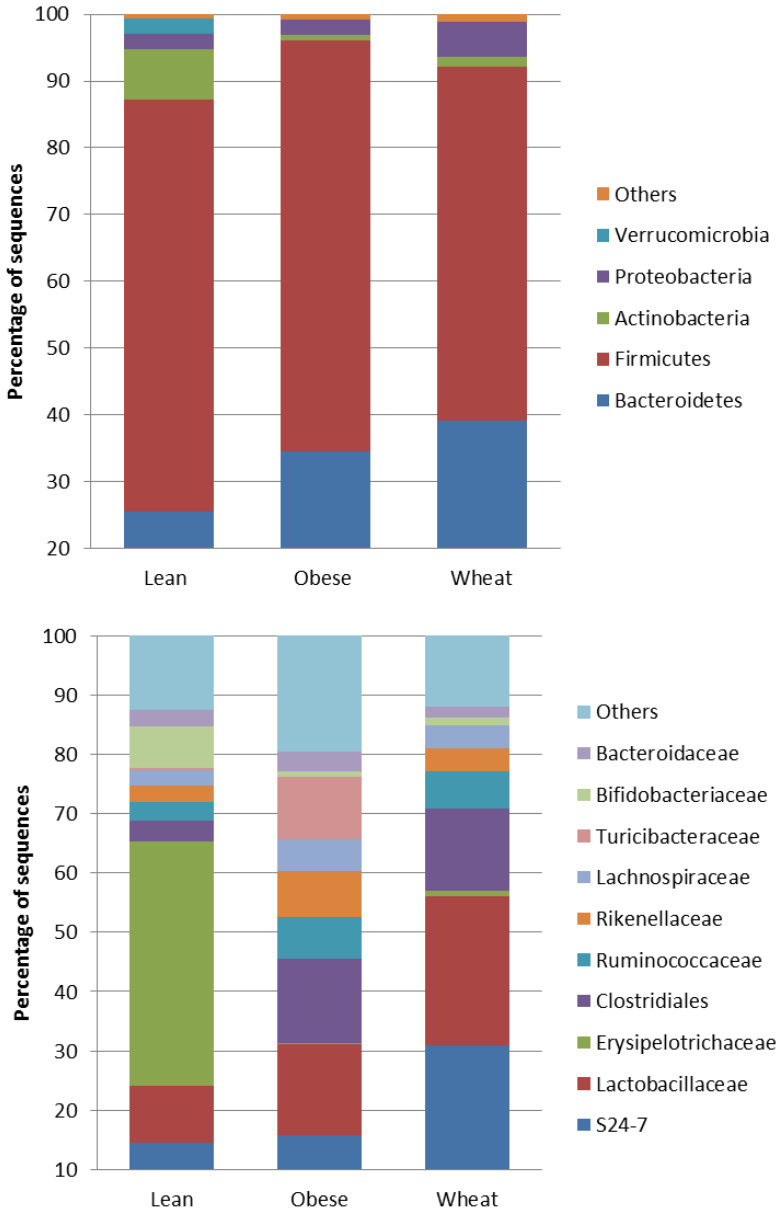
699 **Figures and figure legends**



700

701 **Figure 1 Column chart.** Composition of fecal microbiota in the lean (n=11), obese (n=9)
702 and WW (n=10) group at the phylum (A) and family (B) level. Bars represent average
703 percentage of sequences. To allow for better visualization, the y axis (percentage of
704 sequences) was modified.

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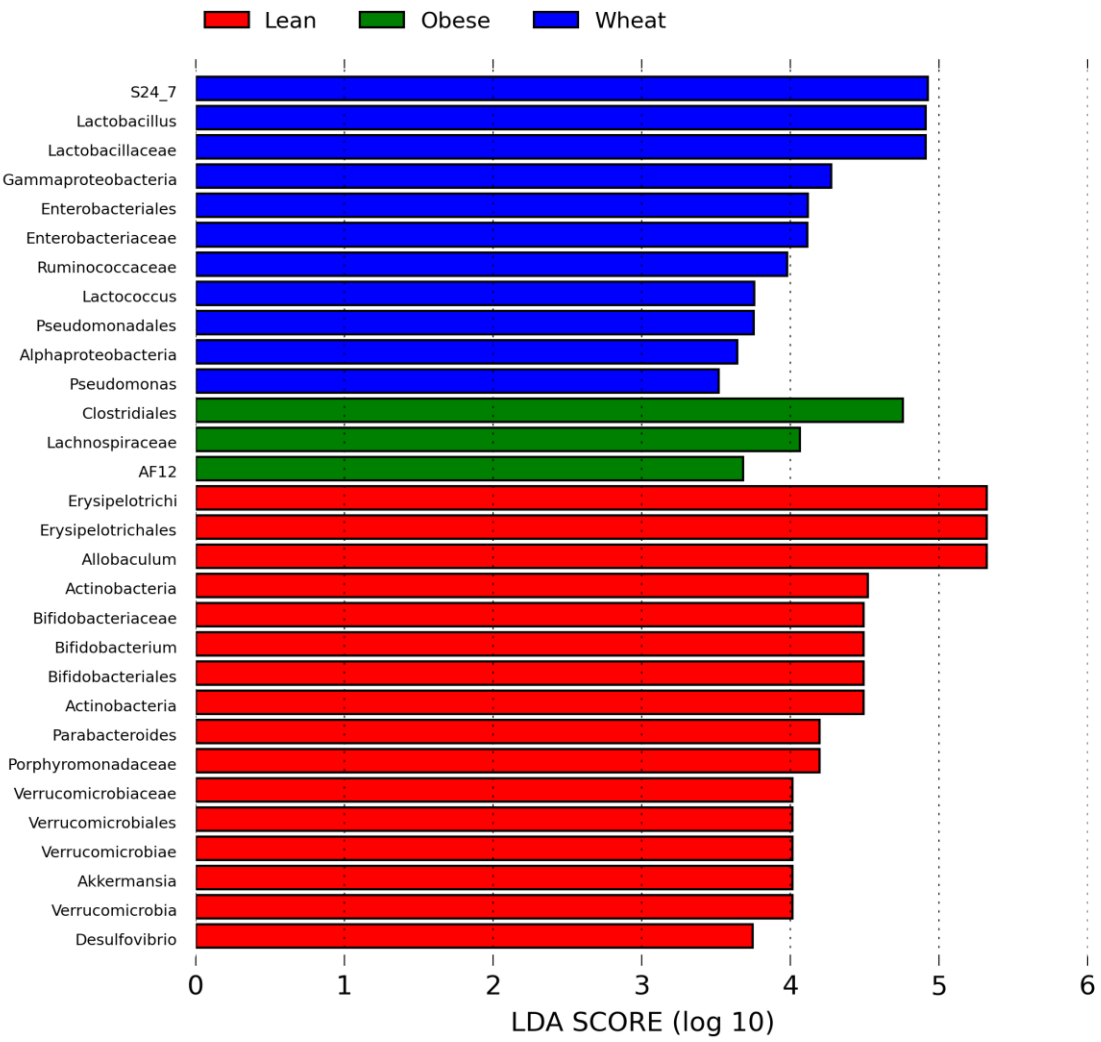

705

706 **Figure 2 LefSe plot.** Bacterial groups that showed statistical significance based on LEfSe.

707 A LDA score threshold of >3.5 was used.

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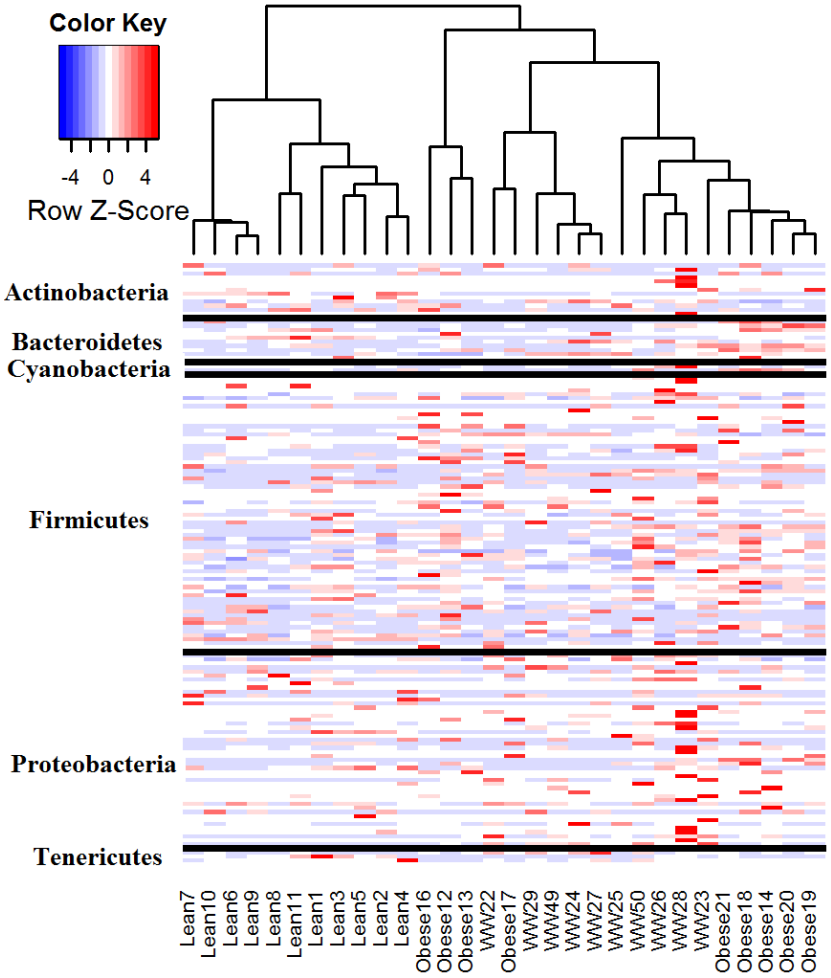

711

712 **Figure 3 Heatmap**. Heatmap of relative abundance of bacterial taxa (y axis, ordered by
713 abundance) in lean (lean 1-11), obese (obese 1-9) and WW (wheat 1-10) group. This
714 analysis shows that lean subjects clustered separately from obese and WW subjects. For
715 better visualization, this analysis was performed using only half the taxa that together
716 comprised >99% of abundance in each sample (clustering of lean subjects was also true
717 when using all taxa, not shown). Clustering was performed using hclust using the
718 heatmap.2 function as implemented in the gplots package (*Warnes et al., 2015*) in R
719 v.3.0.3.

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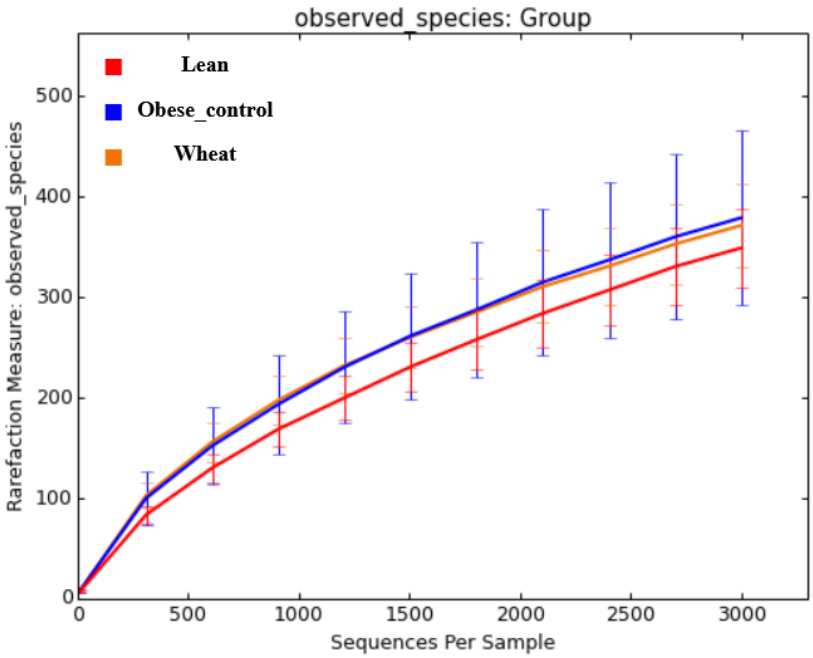

721

722 **Figure 4. Rarefied plot.** This plot shows the relationship between the number of sequences
723 per sample (x axis) and the number of observed species (y axis). This analysis was done on
724 subset of 3000 random sequences per sample. A flat line would indicate that the generation
725 of more sequences will not increase the number of observed species.

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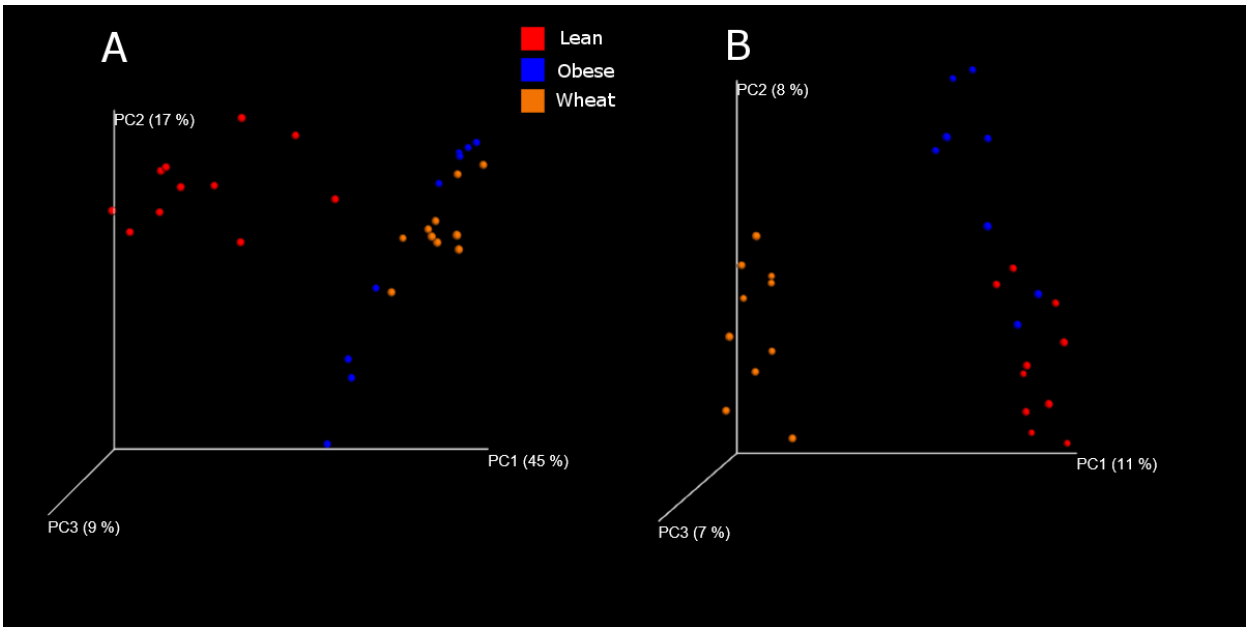

Figure 5 Principal Coordinates Analysis plots. Principal Coordinates Analysis (PCoA) plots of weighted (A, left) and unweighted (B, right) UniFrac distance metrics. There was significant clustering of samples in both weighted and unweighted analysis (ANOSIM, p = 0.001).

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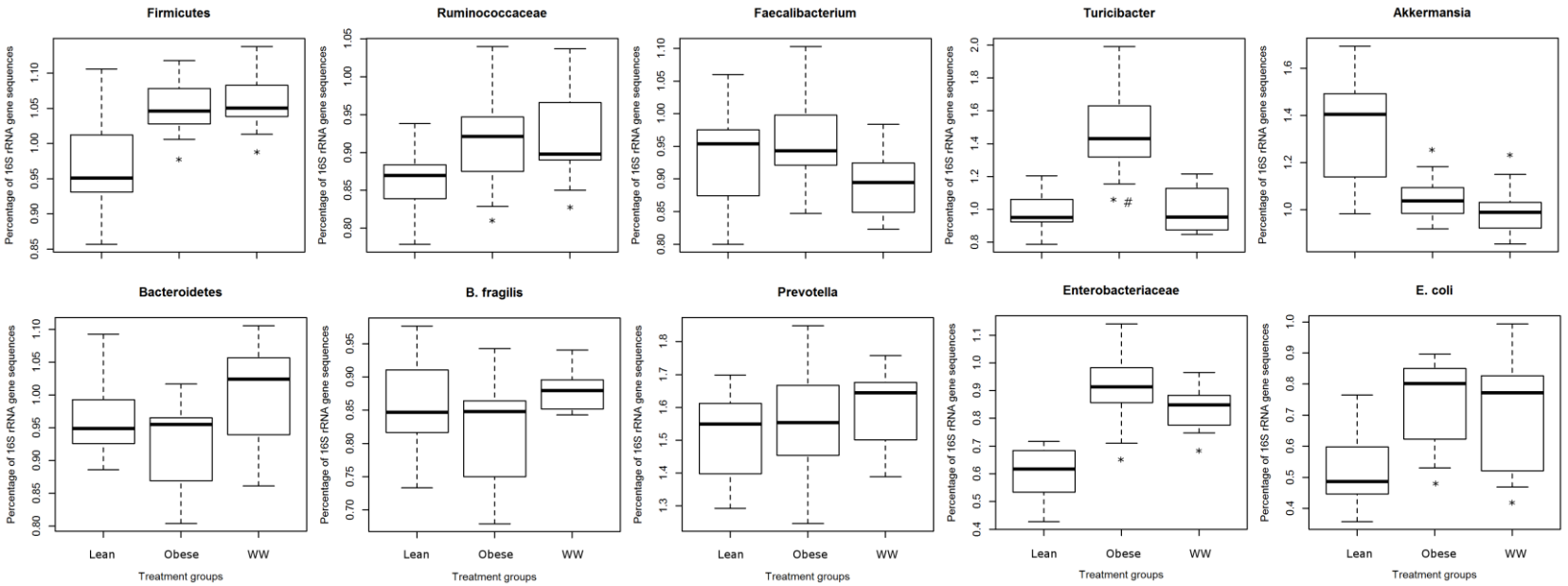

Figure 6 Boxplots. Quantitative real-time PCR (qPCR) results for selected bacterial groups. Results are expressed as relative abundance of 16S rRNA gene sequences (see main text for details). * $p < 0.05$ against lean; # $p < 0.05$ against whole-wheat (WW).

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