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

Jose F Garcia-Mazcorro, Ivan Ivanov, David A. Mills, Giuliana Noratto

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 whole-wheat 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. Short-chain fatty acids were measured in caecal contents using quantitative high-performance liquid chromatography photo-diode array analysis. Results showed no statistical difference in final body weights between the obese-control and the WW group. The bacterial richness (number of Operational Taxonomic Units) 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). Caecal 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 caecal succinic acid was lower in the WW group compared to obese but especially to the lean group. WW consumption was associated with ~3 times higher abundances of *Lactobacillus* spp. compared to both obese and lean control mice. Analysis 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 revealed significant differences in several metabolic features of the microbiota among the treatment groups, including carbohydrate, amino acids and vitamin metabolism ($p < 0.01$, Kruskal-Wallis test). 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.

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

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Abstract

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Diabetic (db/db) mice were fed standard (obese-control) or whole-wheat 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. Short-chain fatty acids were measured in caecal contents using quantitative high-performance liquid chromatography photo-diode array analysis.

Results showed no statistical difference in final body weights between the obese-control and the WW group. The bacterial richness (number of Operational Taxonomic Units) 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). Caecal 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 caecal succinic acid was lower in the WW group compared to obese but especially to the lean group. WW consumption was associated with ~3 times higher abundances of *Lactobacillus* spp.

compared to both obese and lean control mice. Analysis 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 revealed significant differences in several metabolic features of the microbiota among the treatment groups, including carbohydrate, amino acids and vitamin metabolism ($p < 0.01$, Kruskal-Wallis test). 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.

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 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 changes 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 et al., 2015*). Whole-wheat (WW) is often recommended by medical nutritionists as part of a healthy diet for both overweighed and lean individuals. While several investigations have previously addressed

the nutritional benefits of consuming WW (*Stevenson et al., 2012*), very few studies have researched the potential of either WW or its individual nutrients to alter the gut microbiota of lean or obese individuals (*Neyrinck et al., 2011*) or as part of dietary management to treat obesity. One study investigated the effect of replacing refined wheat with whole-grain wheat for 12 weeks on body weight and fat mass in overweighed women (*Kristensen et al., 2011*). This short-period of 12 weeks was enough to significantly reduce percentage fat mass but no body weights. Here we show that an 8-week consumption period of an isocaloric WW diet did not significantly change body weights in obese-diabetic mice. Overall, obese mice under WW-supplemented diet showed similarities to obese controls with regards to gut microbial composition and predicted metabolic profile. The effect of WW was mostly observed on caecal concentrations of butyrate and succinate and a few bacterial groups such as *Lactobacillus*. The results may have implications in clinical dietary management of obesity using WW.

METHODS

Study design

The Institutional Animal Care Use Committee from Washington State University approved all experimental procedures (animal protocol approval number: 04436-001). Two strains of male mice were used in this study, BKS.Cg- + *Lepr^{db}/+Lepr^{db}/OlaHsd* obese diabetic (db/db), and lean BKS.Cg-*Dock7m +/+ Lepr^{db}/OlaHsd* (Harlan Laboratories, Kent, WA). Animals were purchased at 5-6 weeks of age and maintained in ventilated rack system with food and water provided *ad libitum* throughout the study. We received 11 mice for the lean group and 10 mice from all other groups. After 7 days of acclimatization, obese mice were randomly divided into two groups (n=10 each) namely obese (AIN-93 G Purified Rodent Diet) and WW (whole-wheat

supplemented diet). The wild type mice group (n=11) was named lean (AIN-93 Diet). Diets were made by Dyets Inc. (Bethlehem, PA) (Table 1). Four or five mice per cage were housed in an environment-controlled room (23 °C, 12 hours dark-light cycle). All mice were visually inspected every day and body weight was recorded from all animals once a week.

Fecal collection and DNA extraction

Fresh distal colon contents (see qPCR analysis below) and fecal samples were obtained from all mice at the end of the study (8 weeks) and stored at -80 ºC prior to DNA and 16S rRNA gene profiling analysis. Total DNA was extracted from at least two different fecal pellets weighting approximately 200 mg. Following bead-beating, the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) was used for DNA extraction following the manufacturer's instructions. DNA concentration and purity was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to a working concentration of 5 ng/μL.

High-throughput sequencing of 16S rRNA genes

Amplification and sequencing were performed as described elsewhere (*Bokulich et al., 2014*). Briefly, the V4 semi-conserved region of bacterial 16S rRNA genes was amplified using primers F515 (5′–GTGCCAGCMGCCGCGGTAA–3′) and R806 (5′–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 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*).

160

161 **Quantitative real-time PCR (qPCR) analysis**

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

166

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

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

177

178 **Statistical analysis**

179 ANOVA and the non-parametric alternative Kruskal-Wallis test were used to analyze final body
180 weights and SCFAs concentrations, respectively. Multiple-comparisons were performed using
181 Tukey and Mann-Whitney tests. The Bonferroni (for Mann-Whitney tests) and False Discovery
182 Rate (for Tukey's tests) corrections were used to adjust for multiple comparisons. Analysis of

Similarities (ANOSIM) was used to test for clustering of microbial communities using weighted and unweighted UniFrac distance matrices. QIIME v.1.8.0, R v.3.0.3 (*R core team*), PAST (*Hammer et al., 2001*) 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 a LDA score threshold of 3 (*Segata et al., 2011*). STAMP (*Parks et al., 2010*) was used to visualize and analyze the PICRUSt data with ANOVA and False Discovery Rate. Unless otherwise noted, an alpha of 0.05 was considered to reject null hypotheses.

RESULTS

One mouse in the obese group died for reasons unrelated to this study. At the end of the study, there was a significant ($p < 0.01$, ANOVA) 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 ($p = 0.96$, Tukey's test).

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%),Verrucomicrobia (~0.8%) and others (Fig. 1). There was no statistical difference in relative abundance of the two most abundant phyla (Firmicutes and Bacteroidetes), partly

206 because of the high variability among individual mice. The ratio Bacteroidetes/Firmicutes was
207 lower in the lean (median: 38.7%) compared to the obese group (median: 85.3%) and the WW
208 group (median: 75.4%) but this difference did not reach significance ($p = 0.12$, Kruskal-Wallis).
209 Several statistical differences were found in low abundant phyla. Actinobacteria and
210 Verrucomicrobia were higher in lean, Cyanobacteria, TM7 and Tenericutes were higher in the
211 WW group, and Deferribacteres was higher in obese-control ($p < 0.01$, Kruskal Wallis, Fig. 1).
212
213 LEfSe showed statistical significant differences for several microbial groups at lower taxonomic
214 levels (Fig. 2). Among the bacterial groups that showed differences indicating an effect of WW-
215 supplementation include the genus *Lactobacillus*, the class Gammaproteobacteria and the
216 controversial S24-7 family (see Thread in QIIME google group in references) (Fig. 2). Other
217 differences in bacterial abundances suggested that WW-supplementation did not generate a lean-
218 like microbiome. For example, the genera *Bifidobacterium*, *Allobaculum* and *Akkermansia* were
219 higher in lean compared to both obese and WW group (Fig. 2). Also, the family Ruminococcacea
220 was more similar between obese (median: 6.9%) and WW-supplemented mice (5.6%) compared
221 to lean (2.7%). Despite these differences, overall the fecal microbial composition of obese-
222 control and WW mice was more similar to each other compared to lean although WW-
223 supplementation yielded a unique pattern of bacterial abundances that did not necessarily cluster
224 together with all obese samples (Fig. 3).
225
226 **Alpha diversity**
227 There was no significant difference in number of species (OTUs at 97% similarity) and Chao1
228 diversity index. Interestingly, samples from the obese group showed a more disperse distribution

of OTUs (Fig. 4). Rarefied plots of number of OTUs showed that more than the 3000 sequences per sample used in this study are needed to fully describe the fecal microbiota of all mice.

Beta-diversity

Principal Coordinate Analysis (PCoA) of weighted and unweighted UniFrac metrics showed different clustering of microbial communities. Weighted (which takes phylogenetic information as well as sequence abundance into account) metrics clearly showed a different microbial structure in lean individuals compared to obese and WW groups (ANOSIM $p = 0.001$) (Fig. 5). This was expected based on the clustering of lean subjects using relative abundance of sequence reads (Fig. 3). 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. Using a $p < 0.01$ for ANOVA tests in STAMP, several statistical differences were found (Table 3). 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

252 in relative abundance for different bacterial groups (Fig. 6).

253

254 **SCFAs caecal concentrations**

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

259

260 **DISCUSSION**

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

267

268 *Ley et al*. (2005) showed that lean mice have more Bacteroidetes and less Firmicutes compared
269 to obese mice, a finding that has been reported by several other research groups. However, it is
270 important to note that these observations were division-wide (in other words, there was no
271 specific subgroup such as families or genera that were present high or low in abundance) and,
272 more importantly, that other researchers have found either no difference in Firmicutes and
273 Bacteroidetes between obese and lean (*Duncan et al., 2008*) or more Bacteroidetes in obese
274 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 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 sequencing results about the abundance of Firmicutes but not 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 at lower taxonomic levels 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

298 community differences that are due to changes in relative taxon (OTUs) abundance, while
299 qualitative (unweighted) are most informative when communities differ by what can live in
300 them. Most studies report either weighted or unweighted but few report both. In this study,
301 weighted analysis showed a clear separation of lean samples from all samples from the obese and
302 WW groups, suggesting that the numbers of OTUs are an important determinant to separate lean
303 microbiomes from obese individuals with and without WW. In this study, the results of weighted
304 analysis also show that animal genetics was the predominant factor to separate microbial
305 communities. On the other hand, unweighted analysis showed opposite results: lean and obese
306 samples clustered separately from all samples of the WW group, suggesting that WW helped
307 create an environment that favored a phylogenetically different ecosystem. Importantly, the
308 variation explained by the axes is much lower when using unweighted UniFrac. At this point,
309 both methods should be considered for explaining the changes in gut microbiomes in
310 investigations like this study (*Lozupone et al. 2007*). The discrepancy between the results of
311 weighted and unweighted results suggests that an 8-week period of WW consumption helped
312 change the overall environment in the intestinal lumen, thus modulating what can live and
313 proliferate in it (unweighted results). Thus, the different environment could promote changes in
314 the abundance of specific taxa (weighted results), as shown in this study for several bacterial
315 groups. Given that the assessment of microbial diversity is a major component in microbial
316 ecological studies and closely relates to our understanding of health and health deviations, we
317 expect others to start inspecting and reporting both weighted and unweighted UniFrac distance
318 metrics. The use of both metrics has been shown to be useful in various investigations (*Campbell*
319 *et al., 2015; Igarashi et al., 2014; Wu et al., 2010*).
320

Microbial butyrate is essential for colon health and lower concentrations of this fatty acid are usually considered non-optimal for gut health (*Donohoe et al., 2011*). Nonetheless, studies have shown that obese individuals actually have higher fecal butyrate and other SCFAs compared to lean individuals (*Fernandes et al., 2014*), an observation that suggests that both lower and higher butyrate concentrations than normal may be associated with and perhaps aggravate disease. Similarly, obese mice in this current study (with and without WW supplementation) had higher butyrate concentrations in caecal contents compared to lean mice. Butyrate-producers are abundant in the mammalian gut and mainly belong to the family Ruminococcaceae within the Firmicutes (*Louis et al., 2009*). In this study both sequencing and qPCR revealed higher fecal Ruminococcaceae in obese and WW groups compared to lean individuals, thus potentially explaining the higher caecal butyrate concentrations. Another SCFA that deserves attention is succinic acid, which has been shown to increase in rats fed a high-fat diet (*Jakobsdottir et al., 2013*). In this current study, obese mice had lower concentrations of succinic acid and WW-supplementation seemingly helped to drastically decrease it. Unfortunately, far more attention has been paid to butyrate compared to succinate, propionate and other SCFA (*Cheng et al., 2013; Reichardt et al., 2014*).

The assessment of microbial metabolic activity in complex ecosystems is hampered in part by the huge number of microorganisms and the cost of sequencing either whole genomes or transcriptomes. PICRUSt allows a prediction of the metabolic profile using taxa predicted by 16S rRNA gene sequencing. PICRUSt is, however, not exempt of pitfalls: it only uses information for well-defined 16S sequences and the presence of a given set of genes does not tell anything about their functional activity depending on the specific environmental conditions.

Supported by the similarities in abundance of most bacterial groups between 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.

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

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 compared to obese controls that deserve further investigations. These results

may or may not apply to obesity in human patients. Also, our experimental scheme was not designed to address the effect of WW supplementation on lean mice; whether the observed changes in the gut microbiome and metabolite concentrations are irrespective of mice phenotype may warrant further research. The clinical relevance of this present work remains to be determined.

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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565
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591 **TABLES**

592 **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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607 **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		

Table 3. Metabolic features in Lean (n=11), Obese (n=9) and whole-wheat supplemented (n=10) mice that reached statistical significant differences (ANOVA, $p<0.01$ adjusted for False Discovery Rate in STAMP).

KEGG gene categories			Treatment Groups			
			Lean	Obese	Whole-Wheat	
Level_1	Level_2	Level_3	Mean ± st. dev.	Mean ± st. dev.	Mean ± st. dev.	*p* value
Cellular Processes	Transport and Catabolism	Peroxisome	0.15 ± 0.03^{a}	$0.18\pm0.04^{a,b}$	0.23 ± 0.03^{c}	0.001

Environmental Information Processing	Signal Transduction	Phosphatidylinositol signaling system	0.11±0.01[a]	0.08±0.01[b]	0.10±0.01[a,c]	0.002
	Signaling Molecules and Interaction	Ion channels	0.05±0.01[a]	0.02±0.01[b]	0.02±0.01[b,c]	<0.001
Genetic Information Processing	Replication and Repair	Base excision repair	0.50±0.06[a]	0.41±0.03[b]	0.41±0.04[b,c]	0.001
Human Diseases	Infectious Diseases	Tuberculosis	0.18±0.02[a]	0.13±0.01[b]	0.13±0.02[b,c]	0.001
	Cancers	Pathways in cancer	0.07±0.01[a]	0.04±0.00[b]	0.05±0.00[b,c]	<0.001
	Neurodegenerative Diseases	Amyotrophic lateral sclerosis (ALS)	0.02±0.01[a]	0.03±0.01[b]	0.04±0.01[b,c]	<0.001
	Cancers	Renal cell carcinoma	0.03±0.01[a]	0.01±0.00[b]	0.01±0.01[b,c]	<0.001
Metabolism	Carbohydrate Metabolism	Fructose and mannose metabolism	1.16±0.19[a]	0.94±0.08[b]	0.86±0.12[b,c]	0.005
		Pentose phosphate pathway	0.92±0.05[a]	0.86±0.07[a,b]	0.78±0.06[c]	0.002
	Metabolism of Cofactors and Vitamins	Porphyrin and chlorophyll metabolism	0.55±0.15[a]	0.85±0.13[b]	0.66±0.14[a,c]	0.009
		Vitamin B6 metabolism	0.17±0.02[a]	0.20±0.02[b]	0.22±0.01[b,c]	<0.001
	Metabolism of Other Amino Acids	Selenocompound metabolism	0.40±0.02[a]	0.37±0.01[b]	0.35±0.01[b,c]	<0.001
		Cyanoamino acid metabolism	0.23±0.04[a]	0.33±0.02[b]	0.30±0.06[b,c]	0.002
		beta-Alanine metabolism	0.18±0.04[a]	0.22±0.04	0.28±0.06[b]	0.005
	Carbohydrate Metabolism	C5-Branched dibasic acid metabolism	0.23±0.06[a]	0.32±0.02[b]	0.31±0.02[b,c]	0.003
	Biosynthesis of Other Secondary Metabolites	Phenylpropanoid biosynthesis	0.12±0.03[a]	0.20±0.02[b]	0.17±0.05	0.003
	Metabolism of Terpenoids and Polyketides	Biosynthesis of ansamycins	0.14±0.04[a]	0.11±0.02	0.09±0.02[b]	0.006
	Biosynthesis of Other Secondary Metabolites	Flavonoid biosynthesis	0.02±0.01[a]	0.01±0.00[b]	0.00±0.00[b,c]	<0.001
		Stilbenoid, diarylheptanoid and gingerol biosynthesis	0.01±0.01[a]	0.00±0.00[b]	0.00±0.00[b,c]	0.007

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

SCFA	Lean	Obese	Whole-wheat	*p* value
Sodium butyrate	0.97 (0.15-2.65)[a]	2.91 (1.47-4.35)[b]	4.27 (3.05-6.26)[b,c]	<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)[a]	14.60 (8.78-28.01)	9.96 (6.76-12.15)[b]	0.033
Succinic acid	39.84 (15.29-97.63)[a]	22.97 (3.86-71.18)[a,b]	3.12 (0.91-63.36)[c]	0.009

627 Different letters denote statistical significance. *p* values come from the Kruskal-Wallis test and
628 multiple comparisons were performed using the Mann-Whitney test and corrected with the
629 Bonferroni method.
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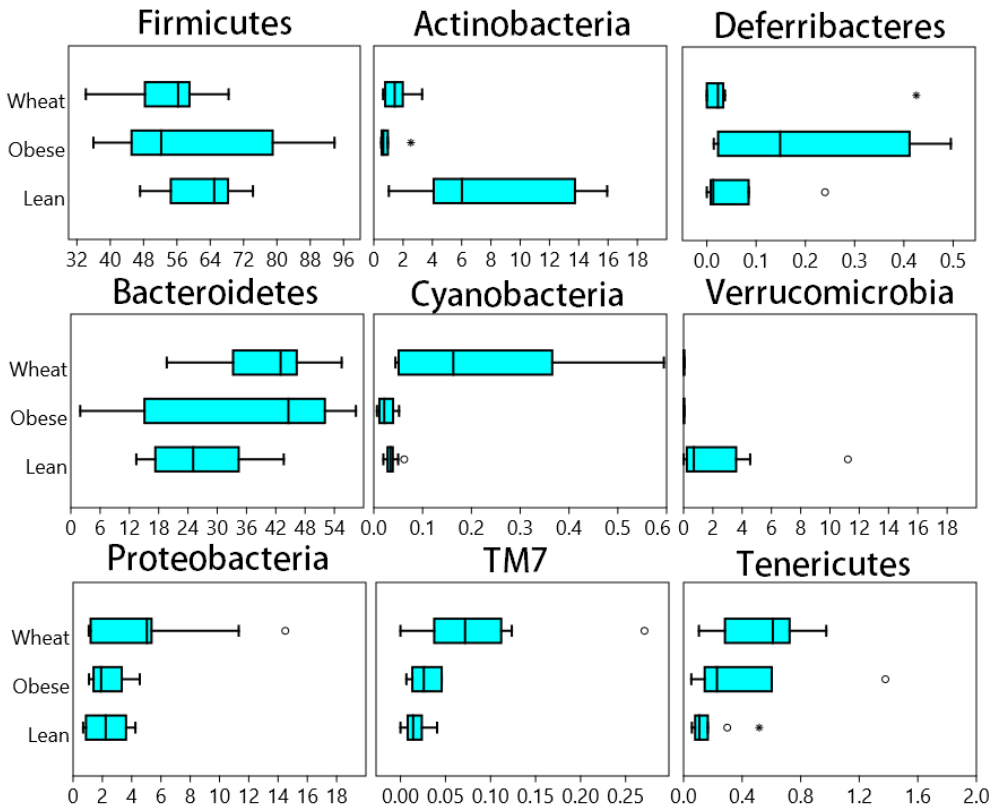
645 **Figures and figure legends**



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647 **Figure 1 Box plots.** Composition of fecal microbiota at the phylum level in the Lean (n=11),
648 obese control (Obese, n=9) and whole-wheat (Wheat, n=10) group. Boxes represent the 25-75
649 quartiles, the median is shown with a vertical line inside the box. Values outside 1.5 times the
650 box height are shown as circles; values outside 3 times the box height are shown as stars. The
651 differences in relative abundance of Firmicutes, Bacteroidetes and Proteobacteria did not reach
652 statistical significance (see main text for details).

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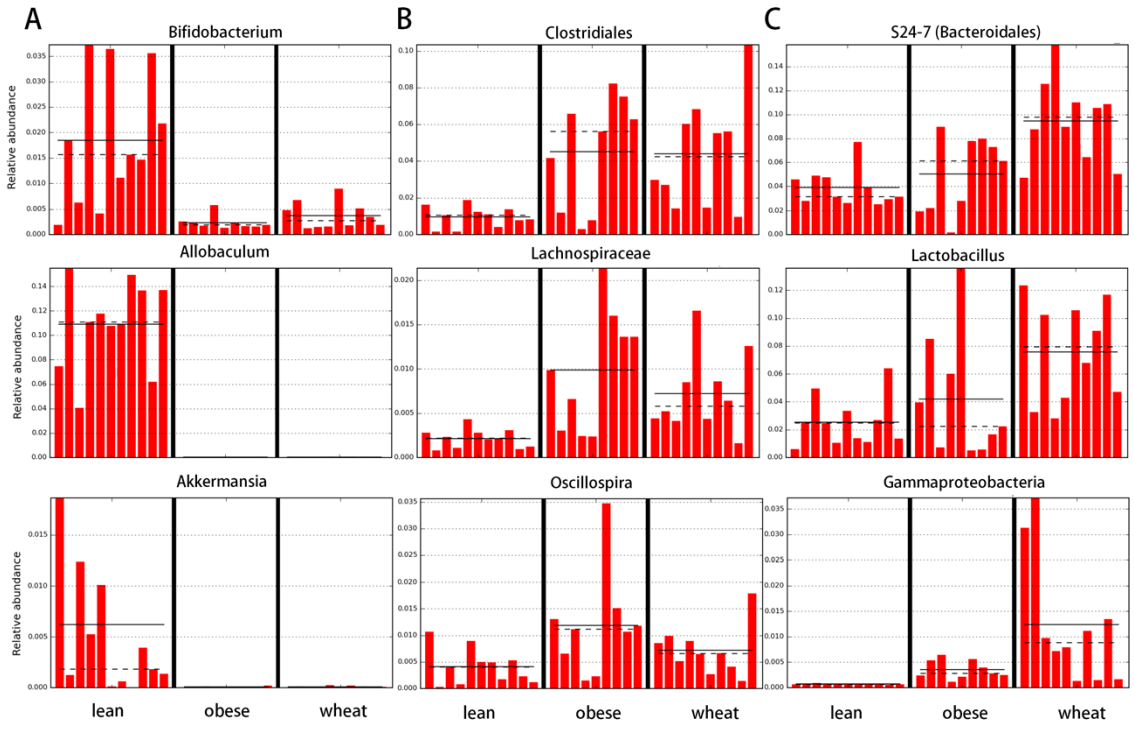

654

655 **Figure 2 Bar charts of most significant results using the LDA effect size method (LEfSe).**

656 LefSe identifies those bacterial groups that showed statistical significance effect size and

657 associate them with the class (in this study treatment group) with the highest median. Panel A

658 (left): lean; Panel B (center): obese-control; Panel C (right): WW-supplemented obese mice.

659 Dotted lines represent medians; straight lines represent averages.

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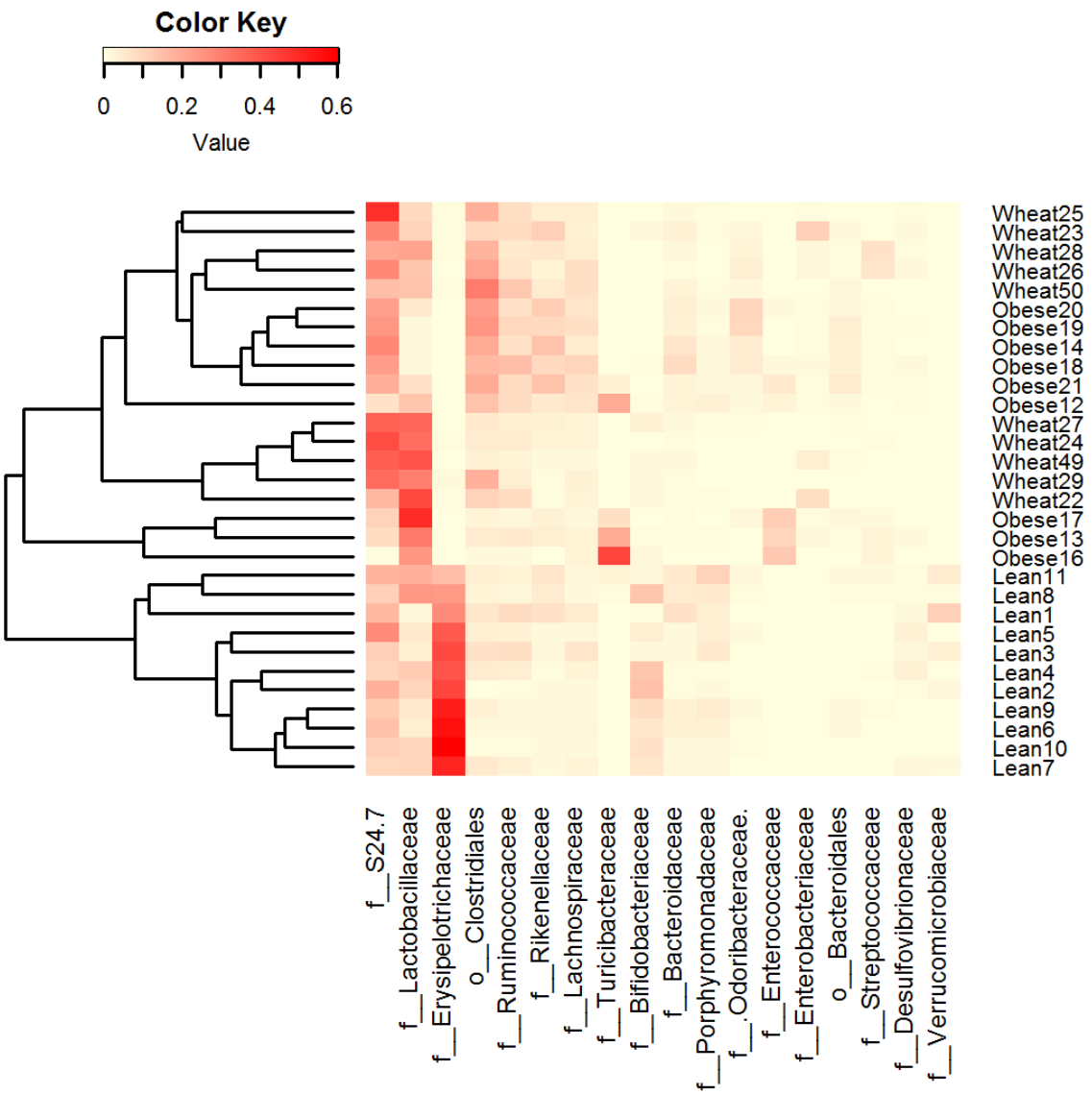

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662 **Figure 3 Heatmap**. Heatmap of relative abundance of the most abundant bacterial taxa at the

663 family level (x axis, ordered by abundance) in Lean (n=11), Obese (n=9) and Wheat (n=10)

664 group. This figure shows that lean subjects clustered separately from obese and WW subjects.

665 Clustering was performed using Bray-Curtis distances in R v.3.2.2.

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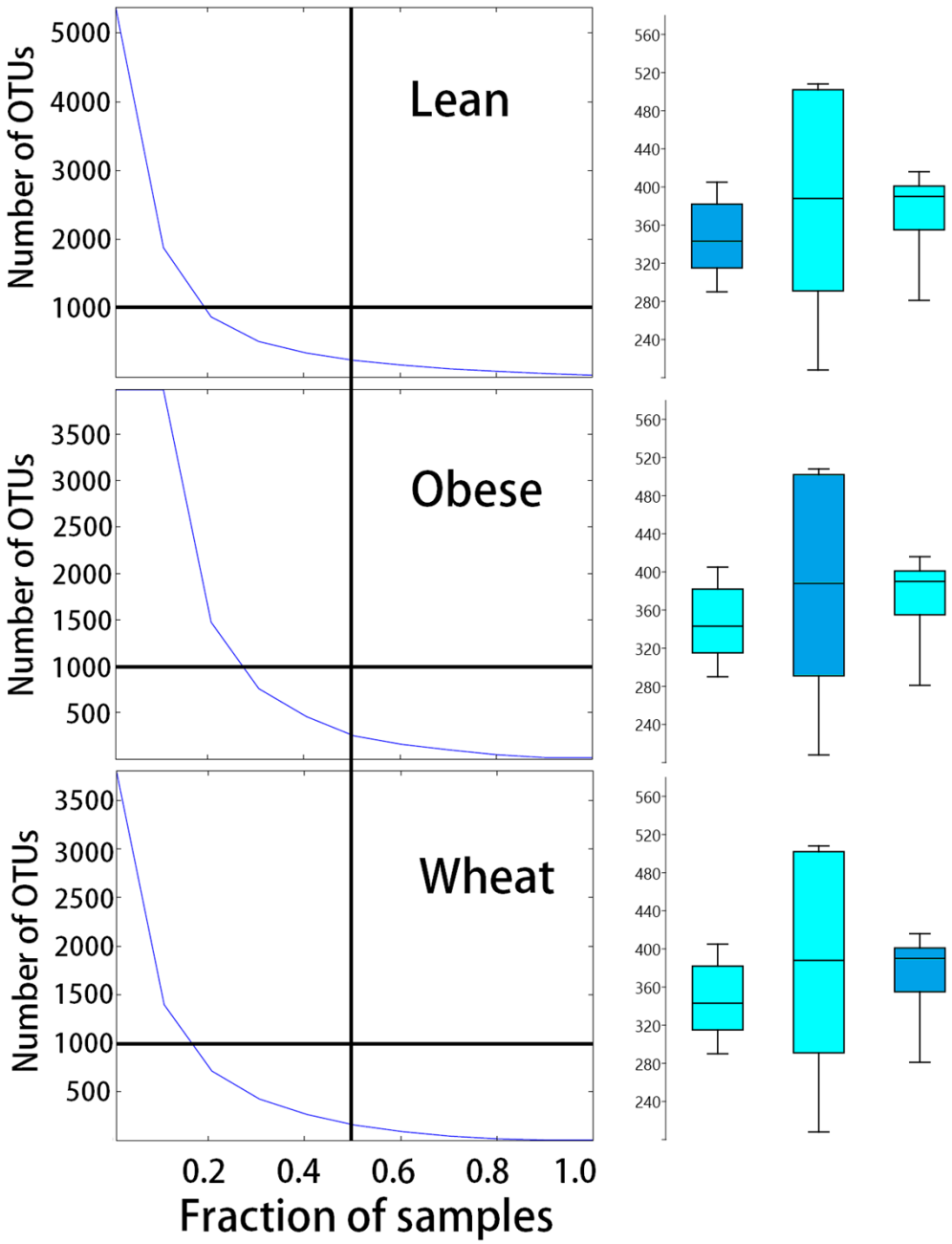

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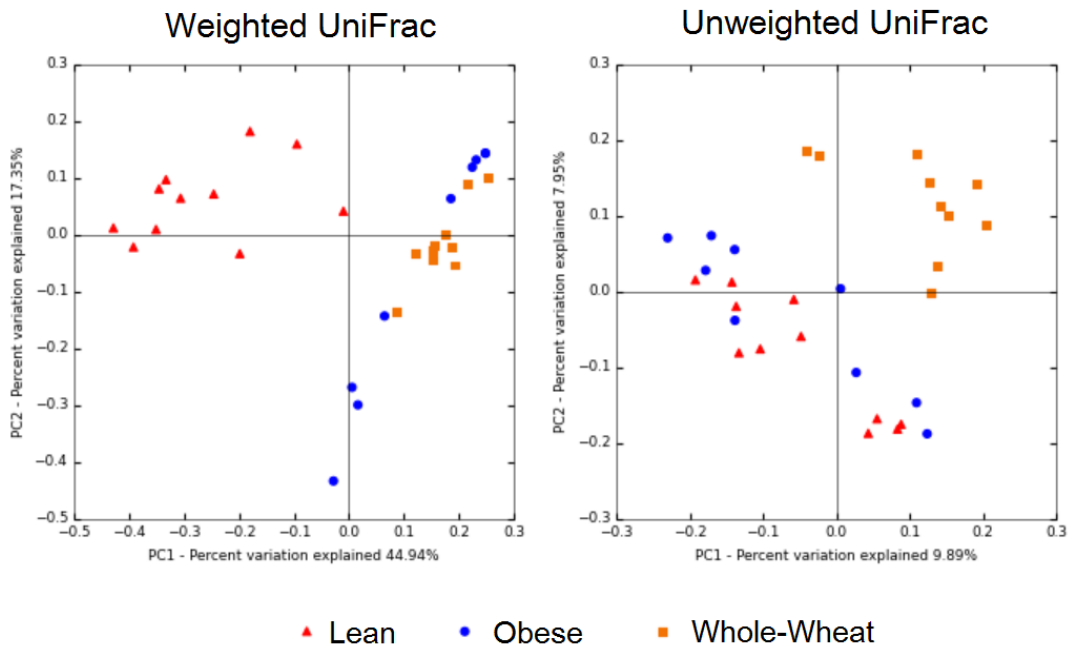
668 **Figure 4 Relationship between numbers of OTUs and the proportion of samples containing**
669 **those OTUs.** Lines were used to illustrate 50% of the samples (vertical line) and 1000 OTUs
670 (horizontal line). Boxplots are shown on the right to illustrate the distributions of the number of
671 OTUs for each treatment group (boxes represent the 25-75 quartiles, the median is shown with a
672 horizontal line inside the box).



673

674 **Figure 5 Principal Coordinates Analysis plots.** Principal Coordinates Analysis (PCoA) plots of

675 weighted (left) and unweighted (right) UniFrac distance metrics. Please note that each plot gives

676 contrasting results with regards to the clustering of samples.

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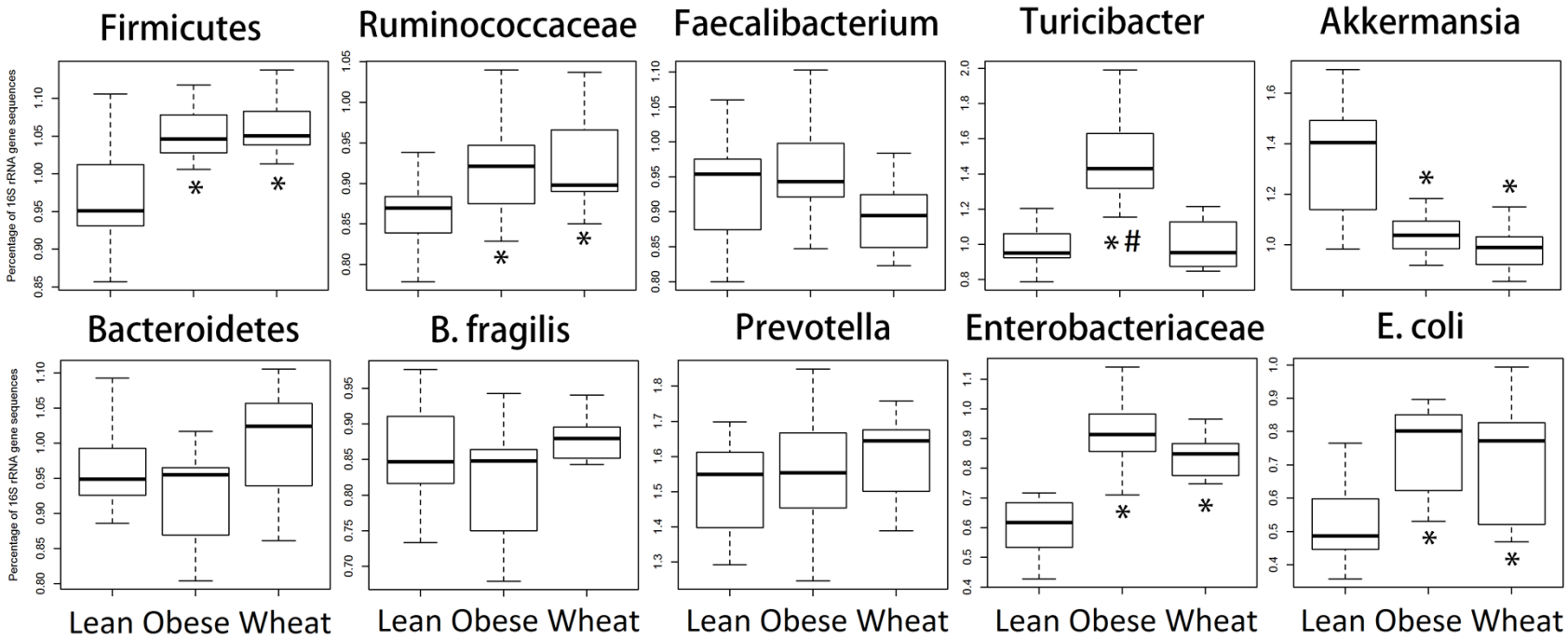

678

679 **Figure 6 Boxplots.** Quantitative real-time PCR (qPCR) results for selected bacterial groups. Results are expressed as relative

680 abundance of 16S rRNA gene sequences (all results were normalized to qPCR data for total bacteria). * $p < 0.05$ against lean; # $p <$

681 0.05 against whole-wheat (WW).