

Influence of whole-wheat consumption on fecal microbial ecology 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 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.

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

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17 **Keywords**

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

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24 **Abstract**

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

32 Diabetic (db/db) mice were fed standard (obese-control) or WW isocaloric diets (WW
33 group) for eight weeks; non-obese mice were used as control (lean-control). High-
34 throughput sequencing using the MiSeq platform coupled with freely-available
35 computational tools and quantitative real-time PCR were used to analyze fecal bacterial
36 16S rRNA gene sequences. SCFAs were measured in feces using HPLC-PDA.

37 Results showed no statistical difference in final body weights between the obese-control
38 and the WW group. Almost 9,000 different bacterial species (Operational Taxonomic Units
39 at 97% similarity) were detected in all mice but the bacterial diversity (number of OTUs)
40 did not differ among the treatment groups. The abundance of Ruminococcaceae, a family
41 containing several butyrate-producing bacteria, was found to be higher in obese (median:
42 6.9%) and WW-supplemented mice (5.6%) compared to lean (2.7%, $p = 0.02$, Kruskal-
43 Wallis test). Fecal concentrations of butyrate were higher in obese (average: 2.91 mmol/mg
44 of feces) but especially in WW-supplemented mice (4.27 mmol/mg) compared to lean
45 controls (0.97 mmol/mg), while fecal succinic acid was lower in the WW group compared
46 to obese but especially to the lean group. WW consumption was associated with ~3 times

47 more relative abundance of *Lactobacillus* spp. compared to both obese and lean control
48 mice. PCoA plots of weighted UniFrac distances revealed a distinctive clustering of lean
49 microbial communities separately from both obese and WW-supplemented mice ($p =$
50 0.001, ANOSIM test). Predictive metagenome analysis using PICRUSt revealed significant
51 differences in several metabolic features of the microbiota among the treatment groups,
52 including fatty acid biosynthesis, carbohydrate and energy metabolism as well as synthesis
53 and degradation of ketone bodies ($p < 0.01$). However, obese and WW groups tended to
54 share more similar abundances of gene families compared to lean mice.

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

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70 **INTRODUCTION**

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

75

76 The mammalian digestive tract is a complex organ that has been constantly co-evolving
77 with trillions of microorganisms (the gut microbiota) to combat environmental pathogens
78 and maximize food digestion for at least 600 million years. Despite its general resilience,
79 the gut microbiota is still susceptible to changes in dietary and other life habits, some of
80 which can lead to imbalances and consequently to disease (*Lozupone et al., 2012*). For
81 instance, substantial evidence has been published showing an association between obesity
82 and changes in gut microbial populations and its metabolism of dietary and endogenous
83 compounds (*Delzenne et al., 2011*). Interestingly, the differences in gut microbial
84 communities between lean and obese individuals are not irreversible (*Turnbaugh et al.,
85 2008*) with diet being the most practical alternative to reestablish microbial equilibrium
86 within the gut. Understanding changes in gut microorganisms in response to dietary
87 modifications is essential to develop effective dietary strategies to help obese patients.

88

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

93 *et al.*, 2015). Whole-wheat (WW) is often recommended by medical nutritionists as part of
94 a healthy diet for both overweighted 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 overweighted
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

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 °C 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'-

139 GGACTACHVGGGTWTCTAAT-3'), with the forward primer modified to contain a
140 unique 8-nt barcode and a 2-nt linker sequence at the 5' terminus. Amplicons were
141 combined into two separated pooled samples and submitted to the University of California
142 Davis Genome Center DNA Technologies Core for Illumina paired-end library preparation,
143 cluster generation, and 250-bp paired-end sequencing on an Illumina MiSeq instrument in
144 two separate runs. For data analysis, raw Illumina fastq files were demultiplexed, quality
145 filtered, and analyzed using the freely available Quantitative Insights into Microbial
146 Ecology (QIIME) Virtual Box v.1.8.0 (*Caporaso et al., 2010*). Operational taxonomic units
147 (OTUs) were assigned using two different approaches: first, using UCLUST v.1.2.22
148 (*Edgar, 2010*) as implemented in QIIME using the open-reference clustering algorithm
149 described in (*Rideout et al., 2014*) for alpha and beta diversity analyses; and second, using
150 the pick_closed_reference_otsu.py QIIME script for further analysis using PICRUSt (see
151 Predicted metabolic profiles below). The Greengenes 13_5 97% OTU representative 16S
152 rRNA gene sequences was used as the reference sequence collection (*DeSantis et al.,*
153 *2006*). Alfa and beta diversity analyses were performed using 3000 random sequences per
154 sample (lowest number of sequences in a sample after demultiplexing, filtering and OTU
155 picking). Raw sequences were uploaded into the Sequence Read Archive at NCBI
156 (accession number: PRJNA281761). The trim.seqs command in MOTHUR (*Schloss et al.,*
157 *2009*) was used for splitting original fastq files per sample for uploading to SRA.
158

159 **Predicted metabolic profiles**

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

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

169

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

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

175

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

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

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

186

187 **Statistical analysis**

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

199

200 **RESULTS**

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

206

207 **Fecal microbiota composition**

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

227

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

230 there were more similarities between obese and WW groups compared to lean although
231 WW-supplementation seemed to output a unique pattern of bacterial abundances (Fig. 1).
232 Among the bacterial groups that showed differences indicating an effect of WW-
233 supplementation include the genus *Lactobacillus*, which was higher in WW-supplemented
234 group (median: 26.1%) compared to lean and obese (medians: 9.7% and 6.9% respectively)
235 ($p = 0.02$, Kruskal-Wallis test). The obese group had higher *Turicibacter* (3.4%) compared
236 to the lean and the WW group (median: 0%) ($p = 0.01$, Kruskal-Wallis test) and this was
237 confirmed using qPCR (see qPCR below). Within the Bacteroidetes, the controversial S24-
238 7 family (see Thread in QIIME google group in references) was found to be higher in the
239 WW group (median: 31.6%) compared to lean (median: 12.1%) and obese (median: 18.8%,
240 $p = 0.003$, Kruskal-Wallis test).

241

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

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 $p =$

276 0.001) (Fig. 5). This was expected based on the clustering of lean subjects using relative
277 abundance of sequence reads (Fig. 2). On the other hand, the qualitative (does not take
278 sequence abundance into account) unweighted UniFrac analysis shows that the microbiota
279 of the WW group clustered separately from the lean and obese groups (ANOSIM $p =$
280 0.001) (Fig. 5).

281

282 **Predicted metabolic profile**

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

292

293 **qPCR assessment of microbiota in distal colon contents**

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

297

298 **SCFAs caecal concentrations**

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

303

304 **DISCUSSION**

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

311

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

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

339

340 Beta diversity metrics are useful to study similarities of microbiomes, which in turn have
341 critical consequences for understanding health and disease processes. *Lozupone et al.*
342 (2007) explains that quantitative beta-diversity measures (weighted UniFrac distances) are
343 better for revealing community differences that are due to changes in relative taxon (OTUs)
344 abundance, while qualitative (unweighted) are most informative when communities differ

345 by what can live in them. Most studies report either weighted or unweighted but few report
346 both. In this study, weighted analysis showed a clear separation of lean samples from all
347 samples from the obese and WW groups, suggesting that the numbers of OTUs are an
348 important determinant to separate lean microbiomes from obese individuals with and
349 without WW. In this study, the results of weighted analysis also show that animal genetics
350 was the predominant factor to separate microbiotas. On the other hand, unweighted analysis
351 showed opposite results: lean and obese samples clustered separately from all samples of
352 the WW group, suggesting that WW helped create an environment that favored a
353 phylogenetically different ecosystem. At this point, both methods should be considered for
354 explaining the changes in gut microbiomes in investigations like this study (*Lozupone et al.*
355 2007). In order to explain the discrepancy between the results of weighted and unweighted
356 results, it is feasible to hypothesize that an 8-week period of WW consumption helped
357 change the overall environment in the intestinal lumen, thus modulating what can live and
358 proliferate in it (unweighted results). In turn, this different environment may have promoted
359 changes in the abundance of specific taxa (weighted results), as shown in this study for
360 several bacterial groups. Given that the assessment of microbial diversity is a major
361 component in microbial ecological studies, and that these assessments closely relate to our
362 understanding of health and health deviations, we expect others to start inspecting and
363 reporting both weighted and unweighted UniFrac distance metrics. The use of both metrics
364 has been shown to be useful in various investigations (*Campbell et al., 2015; Igarashi et*
365 *al., 2014; Wu et al., 2010*).
366
367 Microbial butyrate is essential for colon health and lower concentrations of this fatty acid

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

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

393

394 **SUMMARY**

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

401

402 **Caveats**

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

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

416

417 **Future directions**

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

422

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626 *provided.*

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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 <i>E. coli</i> 16S gene) | Walter <i>et al.</i> 2000 |
| HDA2 | GTATTACCGCGGCTGCTGGCAC | | |
| Bact834F | GGARCATGTGGTTAATTCGATGAT | Bacteroidetes (Phylum) | Guo <i>et al.</i> 2008 |
| Bact1060R | AGCTGACGACAACCATGCAG | | |
| 928F-Firm | TGAAACTYAAAGGAATTGACG | Firmicutes (Phylum) | Bacchetti <i>et al.</i> 2011 |
| 1040firmR | ACCATGCACCACCTGTC | | |
| BifF | GCGTGCTTAACACATGCAAGTC | <i>Bifidobacterium</i> (genus) | Penders <i>et al.</i> 2005 |
| BifR | CACCCGTTCCAGGAGCTATT | | |
| <i>E. coli</i> F | CATGCCCGCTGTATGAAGAA | <i>E. coli</i> | Huijsdens <i>et al.</i> 2002 |
| <i>E. coli</i> R | CGGGTAACGTCAATGAGCAAA | | |
| TuriciF | CAGACGGGGACAACGATTGGA | <i>Turibacter</i> (genus) | Suchodolski <i>et al.</i> 2012 |
| TuriciR | TACGCATCGTCGCCTGGTA | | |
| RumiF | ACTGAGAGGTTGAACGGCCA | Ruminococcaceae (family) | Garcia-Mazcorro <i>et al.</i> 2012 |
| RumiR | CCTTITACACCCAGTAATCCCGGA | | |
| FaecaliF | GAAGGGCGCCTACTGGGCAC | <i>Faecalibacterium</i> (genus) | Garcia-Mazcorro <i>et al.</i> 2012 |
| FaecaliR | GTGCAGCGAGTTGCAGCCT | | |
| Eco1457-F | CATTGACGTTACCCGAGAAGAAC | Enterobacteriaceae (family) | Bartosch <i>et al.</i> 2004 |
| Eco1652-R | CTCTACGAGACTCAAGCTTGC | | |
| V1F | CAGCACCGTAAGGTGGGAC | <i>Akkermansia muciniphila</i> | Collado <i>et al.</i> 2007 |
| V1R | CCTTGCCTTGGCTTCAGAT | | |
| PrevF | CACCAAGGCGACGATCA | <i>Prevotella</i> (genus) | Larsen <i>et al.</i> 2010 |
| PrevR | GGATAACGCCYGGACCT | | |
| Bfr-F | CTGAACCAGCCAAGTAGCG | <i>Bacteroides fragilis</i> | Liu <i>et al.</i> 2003 |
| Bfr-R | CCGCAAACCTTCACAAC TGACTTA | | |

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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).

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| Level_1 | Level_2 | Level_3 | Treatment Groups | | | SS |
|--------------------------------------|-------------------------------------|---|------------------|------------|-------------|-----|
| | | | Lean | Obese | Whole-Wheat | |
| 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 | * |

| | | | | | | |
|--------------------|---|---|-----------|-----------|-----------|-----|
| 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 | Drug metabolism - other enzymes | 0.27±0.04 | 0.34±0.03 | 0.32±0.06 | * |
| Metabolism | Biodegradation and Metabolism | | | | | |
| 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 | Polycyclic aromatic hydrocarbon degradation | 0.14±0.03 | 0.10±0.01 | 0.10±0.03 | * |
| Metabolism | Biodegradation and Metabolism | Lipoic acid metabolism | 0.04±0.01 | 0.05±0.02 | 0.07±0.01 | * |
| Metabolism | Metabolism of Cofactors and Vitamins | | | | | |
| Metabolism | Xenobiotics | Ethylbenzene degradation | 0.03±0.01 | 0.05±0.02 | 0.06±0.02 | * |
| Metabolism | Biodegradation and Metabolism | | | | | |
| 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 |

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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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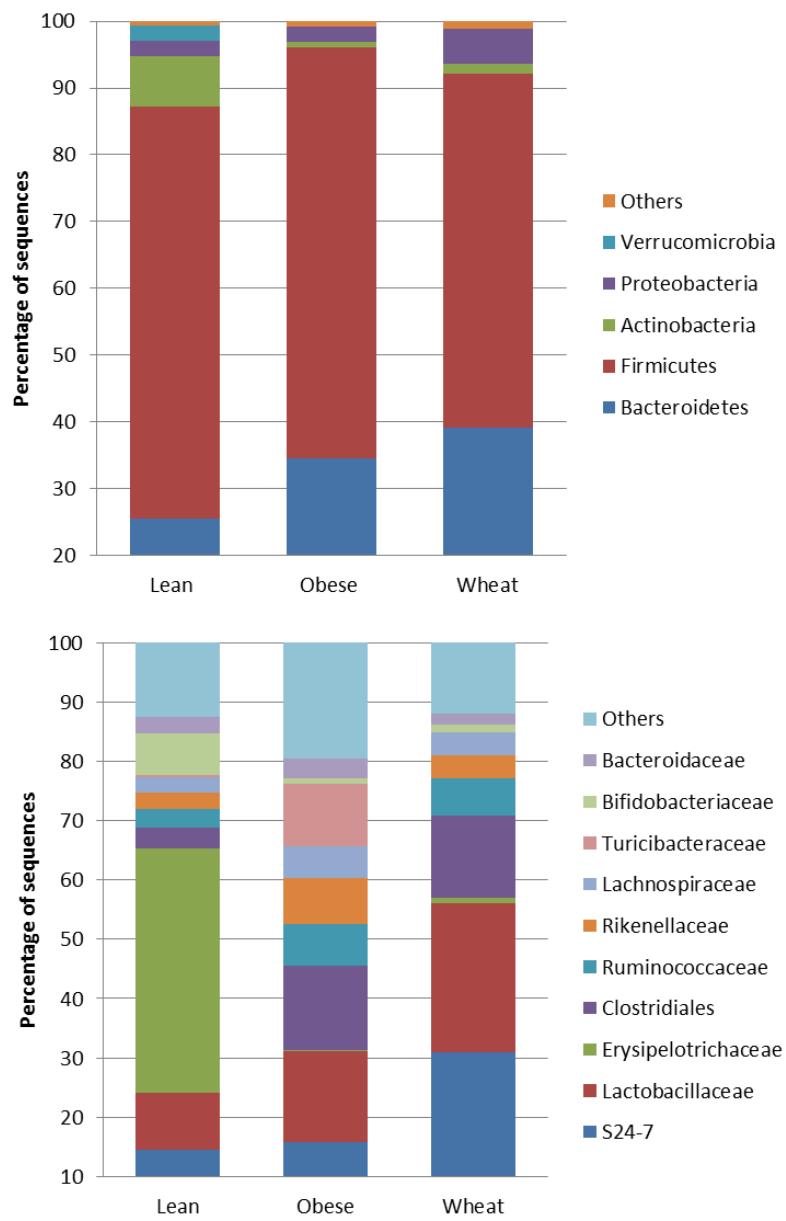
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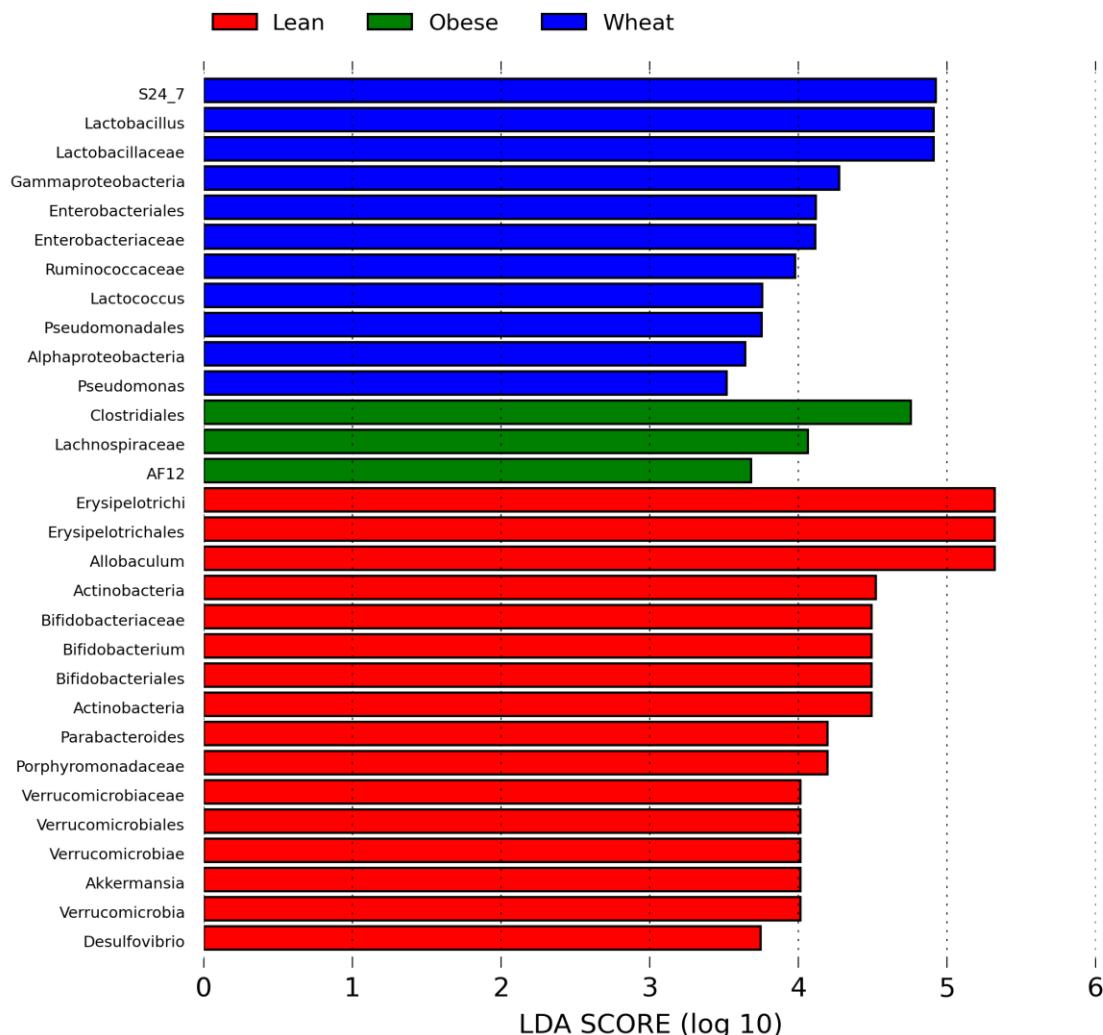
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699 **Figures and figure legends**

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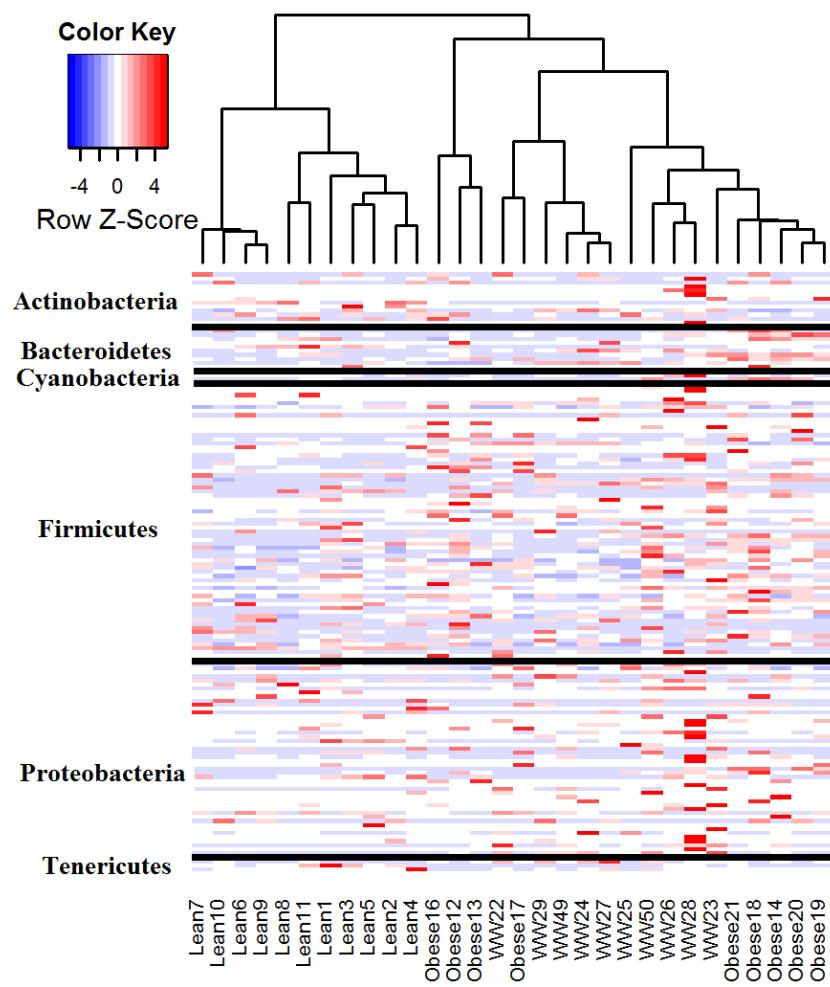
706 **Figure 2 LeFSe plot.** Bacterial groups that showed statistical significance based on LEfSe.

707 A LDA score threshold of >3.5 was used.

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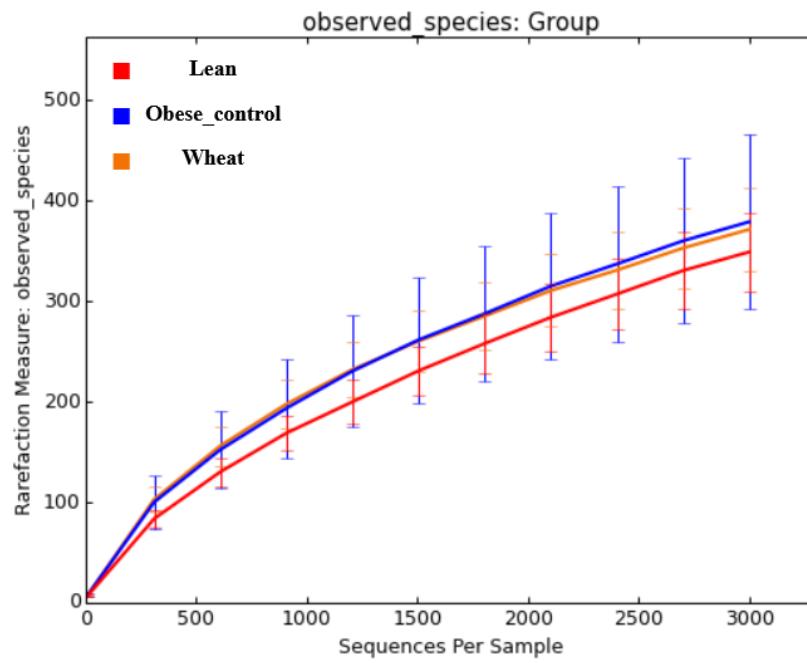
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712 **Figure 3 Heatmap.** Heatmap of relative abundance of bacterial taxa (y axis, ordered by
713 abundance) in lean (lean 1-11), obese (obese 1-9) and WW (wheat 1-10) group. This
714 analysis shows that lean subjects clustered separately from obese 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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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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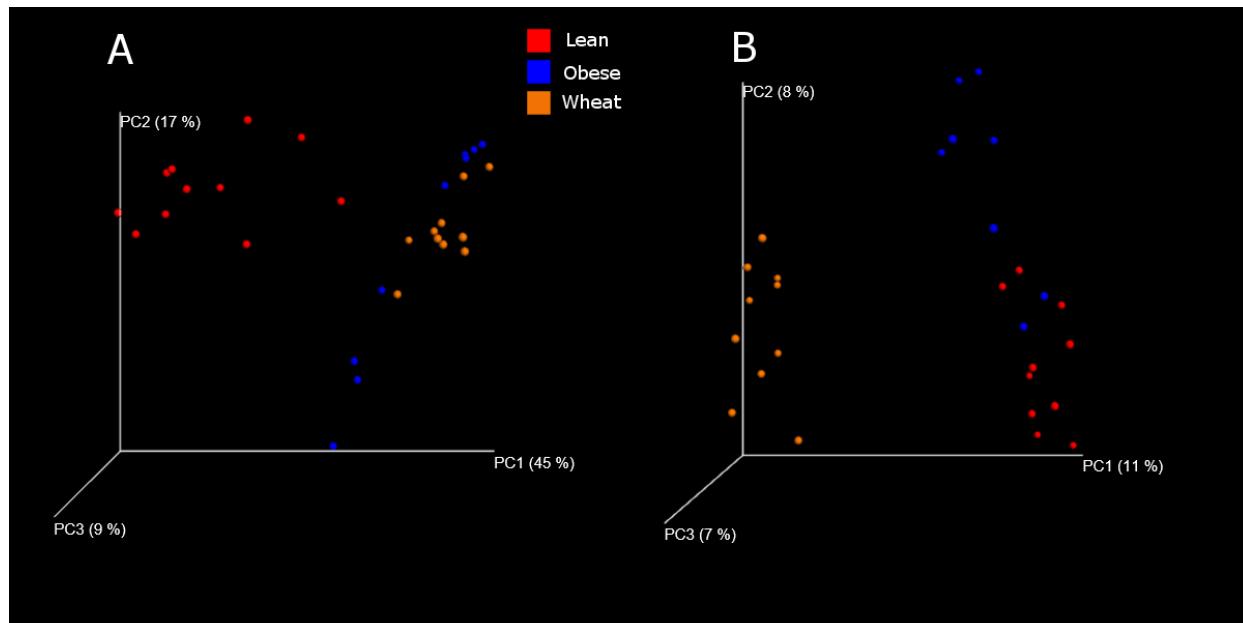
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733 **Figure 5 Principal Coordinates Analysis plots.** Principal Coordinates Analysis (PCoA)
734 plots of weighted (A, left) and unweighted (B, right) UniFrac distance metrics. There was
735 significant clustering of samples in both weighted and unweighted analysis (ANOSIM, $p =$
736 0.001).

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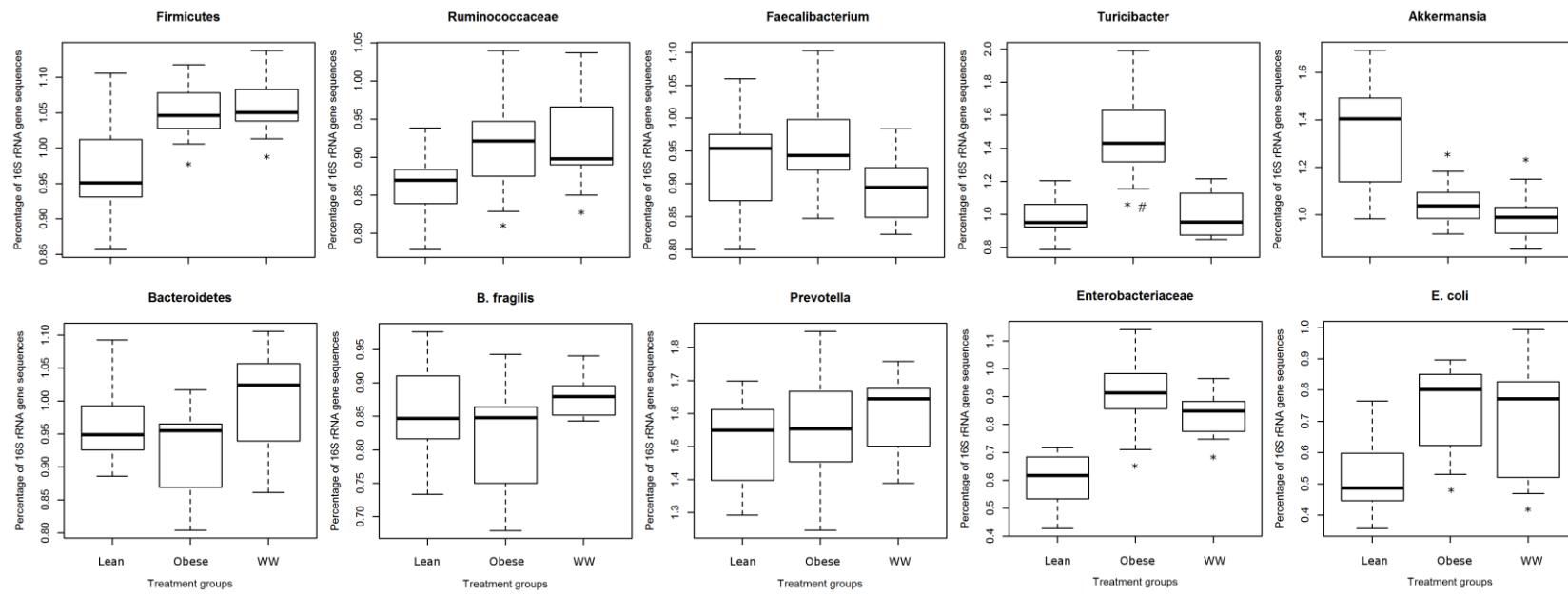
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748 **Figure 6 Boxplots.** Quantitative real-time PCR (qPCR) results for selected bacterial groups. Results are expressed as relative
749 abundance of 16S rRNA gene sequences (see main text for details). * $p < 0.05$ against lean; # $p < 0.05$ against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

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