Effect of dark sweet cherry powder consumption on the gut microbiota, short-chain fatty acids, and biomarkers of gut health in obese db/db mice

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Cherries are fruits containing fiber and bioactive compounds (e.g. polyphenolics) with the potential of helping patients with diabetes and weight disorders, a phenomenon likely related to changes in the complex host-microbiota milieu. The objective of this study was to investigate the effect of cherry supplementation on the gut bacterial composition, concentrations of caecal short-chain fatty acids (SCFAs) and biomarkers of gut health using an *in vivo* model of obesity. Obese diabetic (db/db) mice received a supplemented diet with 10% cherry powder (supplemented mice, n=12) for 12 weeks; obese (n=10) and lean (n=10) mice served as controls and received a standard diet without cherry. High-throughput sequencing of the 16S rRNA gene and quantitative real-time PCR (qPCR) were used to analyze the gut microbiota; SCFAs and biomarkers of gut health were also measured using standard techniques. According to 16S sequencing, supplemented mice harbored a distinct colonic microbiota characterized by a higher abundance of mucin-degraders (i.e. *Akkermansia*) and fiber-degraders (the S24-7 family) as well as lower abundances of *Lactobacillus* and Enterobacteriaceae. Overall this particular cherry-associated colonic microbiota did not resemble the microbiota in obese or lean controls based on the analysis of weighted and unweighted UniFrac distance metrics. qPCR confirmed some of the results observed in sequencing, thus supporting the notion that cherry supplementation can change the colonic microbiota. Moreover, the SCFAs detected in supplemented mice (caproate, methyl butyrate, propionate, acetate and valerate) exceeded those concentrations detected in obese and lean controls except for butyrate. Despite the changes in microbial composition and SCFAs, most of the assessed biomarkers

of inflammation, oxidative stress, and intestinal health in colon tissues and mucosal cells were similar in all obese mice with and without supplementation. This paper shows that dietary supplementation with cherry powder for 12 weeks affects the microbiota and the concentrations of SCFAs in the lower intestinal tract of obese db/db diabetic mice. These effects occurred in absence of differences in most biomarkers of inflammation and other parameters of gut health. Our study prompts more research into the potential clinical implications of cherry consumption as a dietary supplement in diabetic and obese human patients.

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Abstract

Cherries are fruits containing fiber and bioactive compounds (e.g. polyphenolics) with the potential of helping patients with diabetes and weight disorders, a phenomenon likely related to changes in the complex host-microbiota milieu. The objective of this study was to investigate the effect of cherry supplementation on the gut bacterial composition, concentrations of caecal short-chain fatty acids (SCFAs) and biomarkers of gut health using an *in vivo* model of obesity. Obese diabetic (db/db) mice received a supplemented diet with 10% cherry powder (supplemented mice, n=12) for 12 weeks; obese (n=10) and lean (n=10) mice served as controls and received a standard diet without cherry. High-throughput sequencing of the 16S rRNA gene and quantitative real-time PCR (qPCR) were used to analyze the gut microbiota; SCFAs and biomarkers of gut health were also measured using standard techniques. According to 16S sequencing, supplemented mice harbored a distinct colonic microbiota characterized by a higher abundance of mucin-degraders (i.e. *Akkermansia*) and fiber-degraders (the S24-7 family) as well as lower abundances of *Lactobacillus* and Enterobacteriaceae. Overall this particular cherry-associated colonic microbiota did not resemble the microbiota in obese or lean controls based on the analysis of weighted and unweighted UniFrac distance metrics. qPCR confirmed some of the results observed in sequencing, thus supporting the notion that cherry supplementation can change the colonic microbiota. Moreover, the SCFAs detected in supplemented mice (caproate, methyl butyrate, propionate, acetate and valerate) exceeded those concentrations detected in obese and lean controls except for butyrate. Despite the changes in microbial composition and SCFAs, most of the assessed biomarkers of inflammation, oxidative stress, and intestinal health in colon tissues and mucosal cells were similar in all obese mice with and without supplementation. This paper shows that dietary supplementation with cherry powder for 12

47 weeks affects the microbiota and the concentrations of SCFAs in the lower intestinal tract of
48 obese db/db diabetic mice. These effects occurred in absence of differences in most biomarkers
49 of inflammation and other parameters of gut health. Our study prompts more research into the
50 potential clinical implications of cherry consumption as a dietary supplement in diabetic and
51 obese human patients.
52

53 **Introduction**

54 The digestive tract of humans and other animals has coevolved over millions of years with a
55 complex assemblage of many different types of microorganisms (gut microbiota). This
56 coevolution has brought benefits to both forms of life, with the latter sustaining life in the former
57 by helping regulate digestion of nutrients, behavior and the activity of the immune system
58 (*Conlon & Bird, 2014*). Importantly, some bacterial groups in the gastrointestinal tract have
59 become specialized at surviving upon host-derived compounds (*de Vos, 2017*), while other
60 groups are believed to be more dependent on dietary-derived compounds.
61

62 The membership of the gut microbiota (both at the mucus layer and in the lumen) is relatively
63 constant overtime and resilient to change. However, this complex host-microbial ecosystem can
64 also experience extensive variability (both over time within an individual or among different
65 individuals) depending on a variety of factors including the age of the host, dietary patterns,
66 body weight and physical activity. Expectably, diet has strong effects on different aspects of
67 health (*Palmer et al., 2017; Dahan et al., 2017*) and a growing group of researchers have
68 demonstrated that these effects are partly mediated by a change in the composition and/or the
69 metabolic activity of the gut microbiota (*Noratto et al., 2014; Garcia-Mazcorro et al., 2016a and*

2016b; Wu et al., 2011). Although there is still a lot of room for understanding and most studies have only analyzed the fecal microbiota, we now know that dietary modifications can change the composition and activity of the gut microbiota which in turn may promote wellbeing in the host (*Sheflin et al., 2016; Velly et al., 2016*).

Each type of food contain a specific blend of nutrients and other bioactive compounds that can be considered as part of medical strategies to help patients suffering with certain health disorders. Useful examples of these health disorders are obesity, diabetes and associated metabolic conditions (*Via and Mechanick, 2016*), which can be partly treated using foods with more fiber and/or chemical compounds such polyphenols and other anti-oxidants (*Pérez-Jiménez et al., 2010*). These bioactive compounds (i.e. anti-oxidants) have beneficial effects on health and growing evidence suggest that these effects are partly mediated by changes in the gut microbiota (*Noratto et al., 2014; Rowland et al., 2017; Henning et al., 2017*). The benefits of studying this topic are wide and include a better understanding of mechanisms of action thus widening the potential of certain foods to treat specific health disorders.

Cherries are fruits containing bioactive compounds with beneficial properties on human and animal health. Some well-studied cherries compounds include polyphenolics, carotenoids, and tocopherols (*Budak 2017; Mikulic-Petkovsek et al., 2016; Redondo et al., 2017*). Polyphenolics can influence health because of their anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties (*Panche et al., 2016*). Despite the evidence in laboratory animals showing a potential of cherries to help patients with weight disorders (*Wu et al., 2014; Song et al., 2016; Wu et al., 2016*), and the likely involvement of the gut microbiota in this phenomenon

(*Faria et al., 2014*), to our knowledge there are no studies that have investigated the effect of cherry consumption on the gut microbiota using obesity models. Therefore, the objective of this study was to investigate the effect of cherry supplementation on the gut microbiota, SCFAs and biomarkers of gut health using an *in vivo* rodent model of obesity.

Material and methods

Study design

The experimental analyses carried out in this manuscript were approved by the Institutional Animal Care and Use Committee at Texas A&M University (IACUC 2013-0149). Two diets were utilized in this study, one with and one without supplementation with dark sweet cherry (*Prunus avium*) powder (Table 1 and Supplementary Table S1). Both diets were adjusted to contain the same amount of energy (Table 1). Leptin receptor-deficient obese db/db mice (BKS.Cg-+Lepr^{db}/+Lepr^{db}/OlaHsd – fat, black, homozygous) received a diet without cherry supplementation (obese control, n=15) and with 10% cherry powder supplementation (n=15). Lean mice (BKS.Cg-Dock^{7m}+/+Lepr^{db}/OlaHsd – lean, black, heterozygous, n=10) were used as lean controls and fed a standard diet (i.e. without cherry supplementation, Table 1). All mice were purchased from Envigo RMS, Inc. (Houston, TX). Agar based diets were prepared with AIN-93G diet ingredients as reported in detail elsewhere (*Noratto et al., 2016*). The election of an agar-based diet allowed fulfilling the food and part of the water requirement of mice and preserved bioactive compounds in cherry because of the physical properties of agar that remains liquid at 40-45° C. Food and water were provided ad libitum every day for 12 weeks. Food intake and waste were daily recorded. Body weight was recorded once a week and body mass

115 indexes (BMIs) were calculated by dividing body weight (kg) by body length (m^2) at the end of
116 the study (*Jeyakumar et al., 2006*).
117
118 Blood and tissue collection
119 Mice were terminated at week 12 after ~ 12 h fasting by gradual exposure to CO_2 inhalation until
120 the animal became unconscious, followed by cervical dislocation. Blood obtained by cardiac
121 puncture was collected into a tube containing 10 µL of heparin and centrifuged at 10,000 rpm at
122 4° C to obtain blood plasma. Blood plasma samples were aliquoted and frozen at -80°C until
123 analysis. The transverse colon was removed and divided into three sections; one section was
124 fixed in 10% neutral formalin buffer overnight and maintained in 70% ethanol at 4° C for
125 histological analysis of wall layer thickness (see below) while the other two sections were
126 cleaned from intestinal content with 100 mM phosphate buffer solution (PBS, pH = 7) and either
127 kept in RNA later® (Applied Biosystems, Foster City, CA) or subjected to scraping the mucosa
128 off and kept in RNA later® (named colonic mucosal cells). Samples maintained in RNA later®
129 were stored at -80° C for further analysis.
130
131 Bioactive compounds in cherry powder
132 Dietary fiber was quantified by Retch laboratories (Arden Hills, MN) following standard
133 analytical protocols. Total extractable phenolics were extracted as previously reported (*Condezo-*
134 *Hoyos et al., 2014*). Briefly, cherry powder (0.5 g) was homogenized with 3 mL acidic methanol
135 (HCl)/water solution (50:50 v/v, pH 2) and left for 1 h at room temperature and constant shaking,
136 followed by centrifugation at 4000 x g for 10 min at 4° C to obtain the acid methanolic extract in
137 the supernatant. The precipitate was extracted with 3 mL of acetone/water solution (70:30 v/v)

(1:5 ratio, v/v) by agitation for 1 h at room temperature, followed by centrifugation at 4000 x g for 10 min at 4ºC to obtain the acetone extract in the supernatant. The combined supernatants were analyzed for total extractable phenolics by Folin Ciocalteu method (*Condezo-Hoyos et al., 2014*), using a standard curve of gallic acid (0 to 0.2 mg/mL) and expressed as gallic acid equivalents (GAE). The residues were subjected to alkali treatment for extraction of non-extractable or bound phenolics as reported (*Luo et al., 2016*). Briefly, 3 mL of NaOH (4M) were added to residues after extractable phenolics were recovered and maintained in agitation for 2 h under nitrogen atmosphere in a screw capped vial, followed by centrifugation at 4000 x g for 10 min at 4° C. The recovered supernatant was adjusted to pH 2 with HCl (6M) and analyzed for non-extractable bound phenolics using the Folin Ciocalteu method (*Condezo-Hoyos et al., 2014*).

DNA extraction

Colon content and colonic mucosal samples scrapped from terminal colon were collected from all mice at the end of the study and used to purify total genomic DNA using a commercial DNA extraction kit (Zymo Research Corp, Irvine, CA, USA). DNA samples were adjusted to 5 ng/µL and used for two different analyses (high-throughput 16S sequencing and qPCR analyses).

High-throughput 16S sequencing for colonic microbiota

DNA samples extracted from terminal colon contents were used to amplify a small (~300 bp) fragment of the 16S rRNA gene using the primers F515 (5′–GTGCCAGCMGCCGCGGTAA–3′) and R806 (5′–GGACTACHVGGGTWTCTAAT–3′) for further high-throughput sequencing as shown elsewhere (*Garcia-Mazcorro et al., 2016a*). PCR reactions and 16S sequencing were performed at the Molecular Research LP (MRDNA, Shallowater, Texas USA). The MiSeq

instrument (Illumina) was used for sequencing the 16S amplicons following the manufacturer's instructions at MRDNA. This technology has been used in several studies and is recommended by the Earth Microbiome Project (*Caporaso et al., 2012*). Raw 16S data was obtained from MRDNA and analyzed using the freely available bioinformatics pipeline QIIME v.1.8 with default parameters. MRDNA conveniently provides users with files containing joined reads (full.fasta and full.qual files). These files were combined in one single fastq file using QIIME. The resulting fastq file was then used to split sample libraries accordingly to the 8 nucleotide barcodes using the split_libraries_fastq.py in QIIME. Operational Taxonomic Units (OTUs) are operational definitions used to classify 16S rRNA gene sequences from related and unrelated microorganisms and there is debate regarding the best approach to select OTUs from 16S sequences (*He et al., 2015*). In this study we used two approaches to select OTUs. First, we used an open reference algorithm (*Rideout et al., 2014*), which has the advantage of not discarding sequences that do not match the sequence database. The OTU table generated by this approach was used for all diversity and taxonomic analyses. Second, we used a closed reference approach where sequences are discarded if they do not have a close match with the reference sequences. The OTU table generated using this closed approach was used for predicting functional profiles using PICRUSt (see Prediction of metabolic profile below). In this study we used the v. 13_5 of the GreenGenes OTU representative 16S rRNA sequences as the reference sequence collection (*DeSantis et al., 2006*). The phylogenetic method UniFrac (Unique Fraction metric, *Lozupone and Knight, 2005*) was used to investigate differences in microbial communities. Please note that it is important to investigate both quantitative (weighted) and qualitative (unweighted) UniFrac diversity measures because they can lead to different insights into the factors responsible for structuring microbial communities as shown elsewhere (*Lozupone et al., 2007*). All sequence

184 data and associated metadata was uploaded into the Sequence Read Archive at the NCBI
185 (SRP117747).
186
187 qPCR for colonic microbiota and for colonic mucosal samples
188 Unlike high-throughput sequencing (which in this study was only used to analyze the microbiota
189 in colon contents), DNA samples from both colon contents and from colon mucosal samples
190 were used to perform quantitative real-time PCR (qPCR) using primers targeting the 16S rRNA
191 genes for specific groups of microorganisms (Supplementary Table S2) based on the Gut Low-
192 Density Array (GULDA, *Bergström et al., 2012*) approach and other publications (*Garcia-*
193 *Mazcorro et al., 2012; Noratto et al., 2014; Yang et al., 2015*). All qPCR reactions were carried
194 out at Texas A&M University using the described methodology by Bergström et al. (2012) with
195 modifications. Briefly, in this study all assays were ran using a standard curve and these standard
196 curves were constructed using different concentrations of DNA from either the specific
197 microorganisms (*Bacteroides fragilis*, *Lactobacillus plantarum* NRRL No B-4496, *E. coli* NRRL
198 No B-766) or from samples containing high amounts of the desired organism (e.g. a standard
199 curve for Ruminococcaceae was constructed using serial dilutions of a sample with high amounts
200 of Ruminococcaceae DNA as determined by qPCR). DNA samples were adjusted to 5 ng/μL.
201 qPCR data is expressed as log amount of DNA (picograms of amplified DNA) for each bacterial
202 group per 10 ng of total DNA (*Bell et al., 2014*).
203
204 Prediction of metabolic profile
205 Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt,
206 *Langille et al., 2013*) was used to predict the metabolic profile based on 16S sequencing data.

For this analysis, we used the OTU table obtained from the closed reference approach described above. PICRUSt results were visualized and analyzed using STAMP (*Parks and Beiko, 2010*) with default parameters. PICRUSt analysis was performed using the OTU table containing all taxa (full OTU table) and also using filtered OTU tables containing a subset of taxa to explore contributions of different taxa separately.

Short-chain fatty acids (SCFA) analysis

Caecal contents were homogenized with MilliQ water in a proportion of 1:1.5 (weight:volume) and centrifuged at 12000 g for 10 min. Supernatants were then filtered through a 0.45 µm Nylon filter (VWR® Syringe Filters, Houston, TX) and analyzed by high-performance liquid chromatography (HPLC) as reported in detail elsewhere (*Campos et al. 2012; Garcia-Mazcorro et al., 2016a*). Butyric acid, methyl-butyric acid, caproic acid, sodium acetate, sodium propionate, and valeric acid were purchased from VWR and used as standards to quantify their caecal contents based on retention time and area of peaks at $\lambda = 220$ nm.

Histological analyses of colon tissue sections

Paraffin-embedded colon tissues were transversally cut (5 µm thickness) and stained with H&E for microscopic analysis. The thickness of outer colon wall layer was calculated in ImageJ (http://rsb.info.nih.gov/ij/) using 10 measurements (ratio of outer colon wall area to total (outer and inner) colon wall area) from each individual mouse. Photomicrographs were taken with Aperio CS2 digital pathology scanner (Leica Biosystems Inc, Buffalo Grove, IL, USA) and blinded analyzed with regards to treatment group.

230 Endotoxin levels in caecal contents and plasma

231 Caecal contents and blood plasma were subjected to endotoxin analysis using the Endpoint
232 Chromogenic LAL Assay following the manufacturer's protocol (Lonza Walkersville, Inc. –
233 Walkersville, MD). Briefly, caecal contents were weighted, suspended in milliQ water (1:1.5,
234 w:v), centrifuged at 12000 g for 10 min and supernatants transferred to a glass vial for
235 endotoxins quantification. Endotoxin units (EU) were calculated as EU/mg caecal content.

236

237 mRNA levels in colonic tissue and mucosal cells

238 Biomarkers of inflammation, cellular stress, and gut barrier function were analyzed in colonic
239 tissue and mucosal cells. Briefly, tissues or scrapped mucosal cells were mechanically pulverized
240 in liquid nitrogen. RNA was extracted using TRIzol® LS Reagent (Life technologies, Carlsbad,
241 CA) according to the manufacturer's protocol. Purification was carried out with Direct-zol™
242 RNA MiniPrep (Zymo Research Corp, Irvine, CA) according to the manufacturer's protocol.
243 Quantification of mRNA was performed using the ND-1000 spectrophotometer (Nanodrop
244 Technologies, Rockland, DE). Purified mRNA was used to synthesize cDNA using iScript™
245 cDNA Synthesis Kit (BioRad, Hercules, CA). Quantitative real-time polymerase chain reaction
246 (qRT-PCR) was carried out with the SsoAdvanced™ Universal SYBR® Green Supermix
247 (BioRad, Hercules, CA) on a CFX384 Touch Real-Time PCR Detection System (BioRad,
248 Hercules, CA). The reaction volume was 10 µL and all primers were used at a final
249 concentration of 100 nmol/L. The RT-PCR data was analyzed by the 2-ΔΔCT method in
250 reference to ribosomal protein L19 (RPL19) as housekeeping gene (*Schmittgen and Livak 2008*).
251 Primers were purchased from Integrated DNA Technologies, Inc. (San Diego, CA,
252 Supplementary Table S3). Product specificity was examined by dissociation curve analysis.

253

254 Statistical analysis

255 Relative abundances of taxa based on sequencing data, mRNA expression, and qPCR data were
256 compared using the non-parametric Kruskal-Wallis test and multiple comparisons were adjusted
257 using Bonferroni in PAST (*Hammer et al., 2001*). PAST was also used to perform Principal
258 Coordinate Analysis (PCoA) using the weighted and unweighted UniFrac distance matrices
259 obtained from QIIME. The Kruskal-Wallis test was also used for comparison of predicted
260 functional features in STAMP (*Parks and Beiko, 2010*). The non-parametric ANOSIM and
261 Adonis tests were performed for determining whether the grouping of samples by a given
262 category is statistically significant in QIIME. Spearman's correlations matrices featuring data
263 from sequencing analyses, SCFAs, and mRNA levels in colonic mucosal cells identified by
264 Kruskal-Wallis test as significant ($p < 0.05$), were performed using R studio 3.4.0. SCFAs were
265 compared using the Mann Whitney test when comparing only two treatment groups due to lack
266 of detectable values in one group.

267

268 **Results**

269 Cherry powder contributed with phenolics and dietary fiber as bioactive compounds that might
270 reach the lower intestinal tract because of their low bioavailability and most likely modulate
271 microbial populations in the large intestine. Cherry powder had 5.1% fiber and 759 mg
272 GAE/100g of total phenolics (Table 1). A recent study has thoroughly analyzed the profile of
273 phenolics in dark sweet cherry varieties using mass spectrometry and reported approximately 86
274 compounds including phenolics, anthocyanins, flavan-3-ols and flavonols (*Martini et al., 2017*).

275

276 Host physiology

277 Several obese animals died for reasons unrelated to the study (five animals from obese control
278 group, three animals from cherry group), all other mice remained visually healthy throughout the
279 study. Body weight, BMIs, the percentage of adiposity, epidydimal and mesenteric fat as well as
280 liver weight were similar in all obese mice (with and without cherry supplementation) and
281 significantly higher compared to lean controls (Supplementary Table S4). The weight of cecum
282 contents was significantly higher in cherry supplemented mice (314 mg, 198-439 mg, median
283 and interquartile ranges respectively) compared to lean (128 mg, 93-152 mg) and obese controls
284 (191 mg, 104-234 mg) ($p=0.003$, Supplementary Table S4), in part reflecting the higher amount
285 of fiber in the cherry-supplemented diet (Table 1).

286

287 High-throughput 16S sequencing for colonic microbiota

288 High-throughput 16S sequencing allows a deep analysis of complex microbial communities such
289 as the gut microbiota. In this study, the split libraries script yielded a total of 3,171,568 good-
290 quality 16S sequences for analysis (n=32 across all treatment groups, median sequence length:
291 300 nucleotides). The number of sequences per sample varied from 61,284 (lowest) to 142,829
292 (highest). All analyses were performed using a rarefaction depth of 61,000 sequences per sample.

293

294 Overall the colonic microbiota was dominated by six main taxa at the order level representing
295 four phyla: Bacteroidales (phylum Bacteroidetes), Clostridiales and Lactobacillales (phylum
296 Firmicutes), Verrucomicrobiales (phylum Verrucomicrobia), Desulfovibrionales and
297 Enterobacteriales (phylum Proteobacteria). Together, these taxa comprised about 20 different
298 bacterial families which accounted for over 95% of all sequences in most samples (Figure 1).

299 The results of sequencing and/or qPCR showed significant differences in the relative abundance
300 of several members of all these main taxa in supplemented mice.
301
302 The phylum Bacteroidetes contains several bacterial groups associated with health and this group
303 is usually highly abundant in feces and intestinal contents of human and laboratory mice
304 (*Karlsson et al., 2010*). The family S24-7 (a group of fiber degraders) was very similar in lean
305 (median=19.8%) and cherry-supplemented (median=20.3%) and both were about 3 times higher
306 when compared to obese mice (median=7.9%, $p<0.005$ for both comparisons) (Figure 1). While
307 this result supports the observed higher weight of cecum contents in supplemented mice, it also
308 opens up the question of why lean controls (not supplemented) also showed similar levels of
309 S24-7 compared to supplemented mice. The family Bacteroidaceae was similar in all obese mice
310 but only supplemented mice had higher abundance (median=13.5%) compared to lean controls
311 (median=6.6%, $p=0.0124$). This is interesting because *Bacteroides* has been linked to production
312 of SCFAs (*Chen et al., 2017; Rios-Covian et al., 2017*) and at least one group of *Bacteroides* (*B.*
313 *acidifaciens*) has been shown to also use host compounds (*Berry et al., 2013; Sonnenburg et al.,*
314 *2005*) similarly to *Akkermansia*.
315
316 The phylum Firmicutes is also a highly abundant member of the gut microbiota and contains
317 many groups associated with health, for example producers of SCFAs (*Barcenilla et al., 2000*).
318 Interestingly, we did not find any difference in the abundance of the two most abundant families
319 within the Firmicutes: Ruminococcacea and Clostridiaceae. On the other hand, *Lactobacillus*
320 was much lower (~10 times lower) in cherry-supplemented mice (median=0.8%) compared to
321 both lean (median=12.4%) and obese (median=13%) controls ($p<0.005$ for both comparisons), a

finding that was also noticeable at the family level (Figure 1) and that was confirmed using qPCR (see qPCR below).

The phylum Verrucomicrobia is usually low in abundance in the lower gut but it also contains important bacterial groups that have been associated with health such as the mucin-degrader *Akkermansia* (*Derrien et al., 2008*). In this study, the genus *Akkermansia* (family Verrucomicrobiaceae) was lower in obese mice (median=0.07%) compared to lean (median=0.31%, p=0.0402) but especially to cherry-supplemented mice (median=4.9%, p=0.0003), a result that was also confirmed by qPCR. This result was also confirmed using qPCR (see qPCR below).

The phylum Proteobacteria (main order Enterobacteriales) contains bacteria that are usually associated with harmful effects on intestinal health such as several strains of *Escherichia* and *Salmonella*. In this study, the family Enterobacteriacea was more similar between lean (median=0.08%) and cherry-supplemented (median=0.3%) mice and both were much lower compared to obese mice (median=7.3%, $p<0.05$ for both comparisons, Figure 1), a finding that could be considered a positive effect of cherry supplementation. The lower abundance of Enterobacteriaceae in supplemented mice was also confirmed using qPCR (see qPCR below). In this study Enterobacteriaceae was the only family within the order Enterobacteriales but most sequences belonged to an unknown genus. The family Alcaligenaceae (Betaproteobacteria) was higher in supplemented mice (median=1.1%) compared to both lean (median=0.06%) but especially to obese (median=0.01%) controls ($p<0.005$ for both comparisons). There was no significant difference in the abundance of Desulfovibrionales (class Deltaproteobacteria).

345

346 Finally, the family Bifidobacteriaceae (order Coriobacteriales, phylum Actinobacteria) was not
347 detected at all with our sequencing effort. Nonetheless, supplemented mice showed similar
348 abundance of the order Coriobacteriales (median=0.1%) compared to lean (median=0.3%) but
349 only lean mice was higher compared to obese (median=0.08%, $p<0.0001$).

350

351 Alpha diversity analyses

352 The comparison of relative abundances help determine differences in groups of microorganisms
353 (e.g. *Akkermansia*) but it does not help shed light into the diversity of microbial life among the
354 different samples. Interestingly, cherry-supplemented mice showed the highest Shannon
355 diversity indexes (index=8.1) compared to both obese (index=7.4) and lean controls (index=6.9,
356 $p=0.0141$ Kruskal-Wallis). Also, the number of species (OTUs at 97% similarity) was higher in
357 obese controls (5407) and lower in lean (4078), with supplemented mice having intermediate
358 values (4838, $p=0.0078$, Kruskal-Wallis test) but overall the number of OTUs did not reach a
359 plateau for any treatment group, particularly in obese mice with and without cherry (Figure 2).
360 This means that the sequencing effort in this study was not enough to fully describe the total
361 number of species in our samples; however, it is important to remember that these OTU
362 measures were obtained from an open OTU picking approach that does not discard sequences
363 based on matching with reference database.

364

365 Beta diversity analyses

366 The analysis of individual taxa such as *Akkermansia* or *Lactobacillus* yields valuable information
367 about the membership of the bacterial communities; however, the differences in individual taxa

368 may or may not be sufficient to generate a distinctive microbial community. Using weighted
369 UniFrac distances (which takes into account both phylogenetic divergence and the numbers of
370 sequences associated with each OTU), there was a significant clustering of samples according to
371 treatment ($p<0.001$, Adonis test; $p=0.001$, $R=0.716$, ANOSIM test) (Figure 3). There was also a
372 significant difference using unweighted UniFrac ($p<0.001$, Adonis test; $p=0.001$, $R=0.844$,
373 ANOSIM test), which does not take into account the number of OTUs (Figure 3). Please note
374 that these tests often have a low sensitivity (they usually detect a difference when there is none),
375 therefore it is also informative to look at the R values in the ANOSIM test to investigate the
376 strength of clustering (the closest to 1 the strongest the clustering of samples). Therefore, the
377 higher R value in the ANOSIM test for the unweighted UniFrac implies that the clustering is
378 stronger compared to the clustering using weighted UniFrac, meaning that each treatment is
379 mainly associated with phylogenetic distinct bacterial populations rather than the numbers of
380 these populations. This result can easily be appreciated in the PCoA plots of UniFrac metrics
381 (Figure 3).
382
383 Predicted metabolic profile
384 PICRUSt is useful at predicting the metabolic profile of the microbiota based on 16S sequencing
385 data. In this study, a great number of features showed statistical significance among treatment
386 groups (Table 2), especially within metabolism and genetic information processing pathways. In
387 our experience, these differences are not due to stochastic variations (e.g. other studies from our
388 research group have shown no differences in any feature using a very similar methodological
389 approach, *Garcia-Mazcorro et al., 2017*). Interestingly, for almost all features cherry-
390 supplemented mice showed abundances that were in between those abundances in obese and lean

391 controls (Table 2). Lean mice had higher weighted Nearest Sequenced Taxon Index (weighted
392 NSTI) scores compared to obese controls and supplemented mice ($p<0.05$ for both comparisons),
393 meaning that the microbiota of all obese mice was relatively more represented in sequenced
394 genomes.
395
396 The feature with the lowest p value in PICRUSt analysis was associated with terpenoid backbone
397 biosynthesis (adjusted $p=0.0003$), with lean controls having the highest (average: 0.69%) and
398 obese controls the lowest (0.54%) values, with supplemented mice having values in between
399 (0.61%). This topic is interesting because terpene synthases are widely distributed in bacteria
400 (*Yamada et al., 2015*) and terpenes have beneficial properties in human health (*Cho et al., 2017*);
401 however, there is little information about the potential of terpene synthesis in the gut microbiota.
402 In order to investigate what bacterial group was more associated with this difference, we
403 performed PICRUSt on different taxa independently using filtered OTU tables. There was a
404 difference in this feature for the phylum Firmicutes and Bacteroidetes but all obese mice (with
405 and without supplementation) showed very similar abundances compared to lean, suggesting that
406 the group responsible for the overall effect on terpenoid backbone biosynthesis was not a
407 member of either phylum. Interestingly, the independent analysis of Proteobacteria revealed that
408 lean controls had the highest (average: 0.51%) and the obese controls the lowest values (average:
409 0.39%) with supplemented mice somewhere in between (average: 0.45%), a result that is similar
410 to the analysis of all bacterial groups at once. This suggests that a member of Proteobacteria was
411 likely associated with the observed difference in the abundance of genes associated with
412 terpenoid backbone biosynthesis. However, the independent analysis of individual taxa within
413 the Proteobacteria did not yield any useful information with regards to any specific taxa

414 associated with the overall difference in terpenoid backbone biosynthesis, suggesting that this
415 difference was due to the combined contribution of several bacterial groups. This area is indeed
416 worth exploring because terpenoids can work as antibiotics and growing research show that
417 commensal microorganisms can generate potent small molecules (*Modi et al., 2014*). Doing this
418 additional analysis for all features that showed statistical significance (Table 2) is advisable but
419 is outside of the scope of this present manuscript.
420
421 qPCR analyses
422 In this study we performed qPCR analyses using DNA from both colon contents and mucosal
423 samples. Using DNA obtained from samples of colon contents, several qPCR results confirmed
424 the sequencing results (Figure 4). For example, *Lactobacillus* was found to be lower in cherry-
425 supplemented mice compared to both lean and obese controls ($p<0.0001$). Similarly,
426 *Akkermansia* was lower in obese mice compared to lean and cherry supplemented ($p<0.0001$,
427 Kruskal Wallis test) and this result was mainly due to a difference between the obese control
428 group and the cherry-supplemented group ($p<0.0001$) (Figure 4). Also, Enterobacteriaceae were
429 lower in cherry-supplemented and lean mice compared to obese controls. Using qPCR we were
430 able to show that *E. coli* was also lower in supplemented mice, a finding that we could not
431 investigate using sequencing. Moreover, Betaproteobacteria was higher in cherry-supplemented
432 compared to lean and obese controls ($p<0.0001$). Please note that the family Alcaligenaceae
433 within the Betaproteobacteria also showed similar differences using sequencing. qPCR results
434 for colon contents also showed that the abundance of *Bifidobacterium* was higher in
435 supplemented mice compared to obese ($p<0.0001$) and lean ($p=0.002$) controls (Figure 4). These
436 results were partly confirmed using sequencing at higher taxonomic levels (order

Coriobacteriales, see Colonic microbiota above). *C. butyricum* (a butyrate-producing microorganism, *Zhang et al., 2009*) was also found to be higher in supplemented mice (Figure 4).

qPCR analysis of the mucus-associated microbiota helped shed light into an area that is not usually evaluated (most studies evaluate either feces or intestinal contents in part due to ease of sampling and amount of material for analysis). Unfortunately, we only obtained results from four bacterial groups because the results from all other bacterial groups (*Akkermansia* included) were either undetectable or fell below the lowest standard. This can be explained by the fact that commensal bacteria have their habitat in the outer colonic mucus layer, which can be easily lost during tissue dissection and washing (*Johansson et al., 2008*). However, opportunistic pathogens have developed mechanisms to secrete proteases that cleave mucin allowing certain bacteria penetrate and reside in the inner mucus layer (*Pelaseyed et al., 2014*). Interestingly, in this study we found patterns of variations in colon mucosal cells that were not in agreement with those variations observed in colon contents. For example, Betaproteobacteria and Enterobacteriaceae were found to be lower in lean mice compared to all obese mice with and without cherry supplementation (Figure 4). On the other hand and similarly to qPCR results in colon contents, *C. butyricum* was also higher in supplemented mice and Bacteroidetes also showed similar results compared to qPCR results from colon contents (Figure 4).

SCFA in caecal contents

SCFA are microbial metabolites that have been associated with health and disease. In this study, the SCFAs detected in cherry-supplemented mice (caproate, methyl butyrate, propionate, acetate

and valerate) exceeded those concentrations detected in obese and lean controls except for butyrate, either because of higher values or because of higher number of samples in which the SCFAs were detected (Table 3). This is relevant because SCFAs are substrates for colonocytes providing at least 60–70% of their energy requirements (*Suzuki et al., 2008*) with implications for gut barrier function. Based on these results, cherry dietary supplementation contributes with fiber and phytonutrients that apparently promotes a healthier SCFA-producing microbiota, thus contributing to improve colon barrier function and reduce the risk of inflammatory diseases (*Tan et al., 2014*).

Outer colon wall thickness

In this study, the median ratio outer colon wall to total colon wall was higher in supplemented mice (median=0.73) and lean mice (median=0.72) compared to obese controls (median=0.64) but this difference did not reach significance (p=0.08) due to the variability among animal subjects (Supplementary Fig. S1). Increased intestinal concentrations of SCFAs in supplemented mice might have contributed to increase the height of intestinal outer wall layer (*Ramos et al., 1997*).

Biomarkers of inflammation, cellular stress, and gut barrier function in colon tissue and colonic mucosal cells

The colon mucus layers and enterocytes provide the first defense line of the gastrointestinal tract. We have analyzed the mRNA levels of biomarkers of inflammation, cellular stress, and gut barrier function in mucosal cells and colon tissues as a tool to assess whether the changes promoted in gut microbiota by cherry bioactive compounds might also trigger differentiated

responses in the host gate keepers (mucosal layer and epithelial cells) with possible implications in host-bacterial interactions and host immune system. Despite the differences in microbiota and SCFAs, most of the biomarkers analyzed in colonic mucosal cells were similar between experimental groups (Supplementary Table S5), and no difference was found in biomarkers assessed in colon tissues (Supplementary Table S6). In colonic mucosal cells only ATF4 mRNA levels were significantly lower in supplemented group compared to lean ($p<0.05$), and tended to be lower than in obese control. ATF4 is a stress-induced transcription factor whose expression has been correlated with degree of intestinal inflammation and development of inflammatory bowel diseases in adults (*Negroni et al., 2014*). Likewise, VCAM-1, known to control leukocyte/ monocyte intestinal recruitment and localization in LPS-induced inflammation (*Totsuka et al., 2014*), were lower in supplemented group than obese and lean controls, but did not reach significance ($p=0.06$) (Supplementary Table S5).

The concentrations of LPS measured as EU in caecal contents and blood plasma showed no significant difference among experimental groups. However, the LPS concentrations in lumen were not determined due to limitations in sample availability used for DNA extractions and microbiota analysis. Thus, LPS in caecal contents might not necessarily correlate with the LPS concentrations in lumen. We could speculate LPS was lower in supplemented group, thus explaining the lower ATF4 mRNA levels.

Correlation analysis

The separate analyses of microbiota, SCFAs and mRNA levels yielded useful information with regard to the effect of dietary interventions but this data must be integrated in an effort to find

506 possible biologically relevant associations. Overall, data from 16S rRNA sequencing was highly
507 correlated with data from qPCR (Supplementary Figure S2), as discussed above. Interestingly, a
508 high positive correlation was observed between *Bacteroides* and *Akkermansia*, while a negative
509 correlation between *Bacteroides* and *Lactobacillus* was observed. It is also worth mentioning
510 that samples from supplemented mice were associated with higher levels of SCFAs and more
511 *Akkermansia*, an interesting relationship given the production of SCFAs by this bacterial group
512 (*Belzer and de Vos, 2016*).
513
514 **Discussion**
515 Diabetes and obesity are complex diseases that can often be treated using a combination of
516 medications, dietary modifications and physical exercise. Cherries contain fiber and bioactive
517 compounds such as polyphenolics that can promote wellbeing in the host. This study describes
518 the effect of cherry consumption on the colonic microbiota, short-chain fatty acids, and
519 biomarkers of intestinal health using an *in vivo* model of genetic obesity.
520
521 The metabolism and pharmacokinetics of cherry bioactive compounds inside the host are
522 important to evaluate any possible effect of a dietary intervention with cherry. A recent study
523 showed evidence suggesting an involvement of glucose transporters in the small intestine (such
524 as the sodium-dependent linked transporter) in the absorption of anthocyanins from bilberries but
525 it also highlighted the wide differences in bioavailability among different types of anthocyanins
526 (*Baron et al., 2017*). The amount and chemical characteristics of any post-digestion bioactive
527 compounds that reach the large intestine also varies depending on several factors. Importantly,
528 these bioactive compounds are often transformed throughout the digestive tract and reach the

529 lower intestine in a modified form (*Stalmach et al., 2010*). It has been shown that the amount of
530 material reaching the colon is considerable and some authors even catalogue some of these
531 compounds as prebiotics because of its effect in the abundance of certain microorganisms (*Cires*
532 *et al., 2017*). Please note that prebiotics are historically considered to be non-digestible fiber and
533 that the increase in abundance of a certain group of microorganisms (e.g. *Lactobacillus*) when
534 exposed to anti-oxidants may or may not involve direct feeding on the compounds such as in the
535 case of dietary fiber. This is further complicated in case of cherries which contain both fiber and
536 considerable amounts of polyphenolics (*McCune et al., 2011; Wang et al., 2017*).
537
538 This study showed strong evidence that cherry supplementation can modify the colon
539 microbiota, a phenomenon that may be related to the fiber and/or to any post-digestion bioactive
540 compounds reaching the lower intestinal tract. For example, this study showed that the levels of
541 *Akkermansia* spp. in colon contents were higher in supplemented mice compared to both obese
542 and lean controls. *Akkermansia* is a common and relatively abundant (~1%) anaerobic member
543 of the gut microbiota (*Derrien et al., 20008*) that is supposedly highly specialized in host-
544 compounds that may not compete with the microbiota in the highly populated lumen and
545 therefore do not depend on nutrients from host food consumption (*Derrien et al., 2011*).
546 *Akkermansia* indeed deserves attention because of its potential role as mediator of improved
547 inflammatory and metabolic phenotype of mice (*Caesar et al., 2015*). Interestingly, it has been
548 shown that the abundance of *Akkermansia* is lower in the intestinal epithelium of patients with
549 Inflammatory Bowel Disease (*Png et al., 2010*) and in feces often negatively correlates with
550 body weight in rodents and humans (the higher the body weight the lower the abundance of
551 *Akkermansia*, *Everard et al., 2013*). Another study showed that human subjects with higher *A.*

552 *muciniphila* abundance in feces exhibited the healthiest metabolic status (*Dao et al., 2016*).
553 Therefore, members of this taxon have been suggested as biomarkers for a healthy intestine (*Png*
554 *et al., 2010; Swidsinski et al., 2011*). Accordingly, in this study we showed that cherry
555 supplementation was associated with increases the abundance of this health-bearing
556 microorganism, suggesting a beneficial effect of cherry consumption on health. Other similar
557 studies from our research group have also showed that obese mice have less *Akkermansia*
558 compared to lean and quinoa-supplemented obese mice (*Garcia-Mazcorro et al., 2016a*), and
559 others have shown that *Akkermansia* is higher during prebiotic administration (*Everard et al.,*
560 *2011; Van den Abbeele et al., 2011*).
561
562 The reasons behind any increase or decrease of bacterial groups in colon contents are often
563 difficult to clarify. While there may be several explanations for this phenomenon, in the case of
564 *Akkermansia* it has been shown showed that the accompanying microbiota composition
565 determines the magnitude and pattern of host-compounds foraging by this group (*Berry et al.,*
566 *2013*), an interesting phenomenon that has also been shown in other bacteria such as
567 *Bifidobacterium* (*Klaassens et al., 2009*). Importantly, it has been shown that *Akkermansia* is
568 actually composed by at least eight different species based on the 16S rRNA gene (van Passel et
569 al., 2011). This heterogeneity may explain why in other studies the abundance of *Akkermansia*
570 was not necessarily related with health status (*Garcia-Mazcorro et al., 2016b*; *Noratto et al.,*
571 *2014*). In fact, *Akkermansia* was shown to be increased in mouse studies of dextran sodium
572 sulfate (DSS)-induced colitis (*Berry et al., 2012; Kang et al., 2013; Hakansson et al., 2015*) and
573 can seemingly aggravate *Salmonella enterica* Typhimurium-induced gut inflammation in a
574 gnotobiotic mouse model (*Ganesh et al., 2013*). This phenomenon has been explained by an

outgrowth of *Akkermansia* in response to the thickening of the mucus layer (*Ottman et al., 2017*) but it fails to explain the rise of this group in intestinal contents. Given the potential existence of different species of *Akkermansia* (*van Passel et al., 2011*), it is fair to speculate that at least some of these species can use dietary substrates (instead or in addition to host-compounds) and proliferate in the intestinal lumen, thus explaining the rise in numbers. On the other hand, feces also contain abundant mucus (*Swidsinski et al., 2008*), therefore it is also possible that the overgrowth of *Akkermansia* in the mucus layer simply leaked into the lumen.

Other bacterial groups aside *Akkermansia* deserve attention. For example, this study also showed that a group of fiber degraders (the S24-7 family) was also higher in the supplemented group, a finding likely related to the fiber contributed by the cherry-supplemented diet. The genomes of several members of this S24-7 family have been recently explored showing that it contains three trophic guilds, each broadly defined by differential abundances of enzymes involved in the degradation of specific carbohydrates (plant, host and α–glucan) (*Ormerod et al., 2016*). Interestingly, cherry consumption was also associated with lower levels of *Lactobacillus* (a commonly health-bearing group of bacteria) and Enterobacteriaceae (a group comprising potential pathogens). While the reason behind these changes are likely related to the complex microbial-host milieu, a recent study showed that these two groups (both *Lactobacillus* and Enterobacteriaceae) were positively correlated to high levels of *Salmonella*-induced inflammation (*Borton et al., 2017*).

This study showed that the differences in bacterial populations in the supplemented group were accompanied by concentrations of SCFAs that generally exceeded those concentrations in lean

598 and obese controls except for butyrate, an interesting finding because it is precisely butyrate that
599 has caught more attention from the scientific community. Butyrate is produced and also
600 transformed by the gut microbiota; however in our study the increased concentrations of methyl
601 butyrate (a methyl ester of butyric acid) in the supplemented group might be associated with the
602 presence of this ester in cherries as occurs in many plant products. This might be an advantage
603 over production of butyrate because the latter is rapidly metabolized and has limited clinical
604 efficacy in contrast with methyl butyrate which is less polar and less susceptible to being cleared
605 by the body (*Khan et al., 2016*). On the other hand, methyl butyrate may also be produced as a
606 by-product of bacterial metabolism. For example, in one study high production of methyl
607 butyrate was the most significant change induced upon prebiotic and synbiotic supplementation
608 in fecal fermentation *in vitro* (*Vitali et al., 2012*). Here it is also important to highlight the
609 difficulties at determining which bacterial group contributes to each SCFA because multiple
610 groups are often involved in the production/degradation of chemical compounds in the gut
611 (*Pryde et al., 2002*).
612
613 Here we show that cherry bioactive compounds (including fiber) modifies the colonic microbiota
614 and SCFA in obese diabetic mice and we hypothesized that this modification may trigger
615 changes in the gut immunity and physiology of the host. However, here we also showed that
616 cherry powder supplementation did not affect mRNA expression of several biomarkers
617 associated with gut health in spite of a change in bacterial composition and biochemistry. Only
618 ATF4 mRNA levels were different among experimental groups and downregulated in cherry-
619 supplemented mice. ATF4 can be upregulated in response to bacterial LPS as adaptive response
620 that triggers the expression of inflammatory cytokines (TNF-α, IL-6, and IL-10), signaling

pathways (NF-kB and MAPK), and the ATF4-CHOP apoptotic pathway (*Rao et al., 2013*). Therefore, downregulation of ATF4 in supplemented group might be linked to lower levels of bacteria producing LPS in the colon mucus layer, with implications for intestinal cells inflammation and survival. The LPS concentrations in caecal contents might not resemble LPS in lumen layer in contact with the colon mucus layers. Unfortunately, this could not be confirmed due to the limitations intrinsic to the collection of specific fractions of lumen for LPS determination.

In addition, the lower levels of ATF4 and VCAM-1 may also be mediated by the higher SCFAs production in supplemented mice as demonstrated by Huang et al. (2017) *in vitro*. SCFAs inhibited oxidative stress, inflammatory response, and cell adhesion molecules induced by LPS and glucose through activation of their specific G protein-coupled receptors 43 (GPR43) (*Huang et al., 2017*). Even though our experimental conditions did not compromise gut integrity and barrier function as confirmed by LPS in plasma that was similar among experimental groups, our study has provided insights to future studies investigating cherry intake within the context of acute and chronic intestinal inflammation. The improvement in intestinal integrity by polyphenolics enriched extracts, independently of alterations in gut microbiota, was demonstrated over a period of only four weeks in pigs induced subclinical chronic inflammation with *E. coli* LPS injections (*Liehr et al., 2017*). Because the observed changes in bacterial composition and SCFAs could have been related to bioconversion of cherry compounds, it would be interesting to study the effect of cherry compounds in germ-free mice, with the obvious disadvantage of not representing a real-life scenario. In general, our results reveal interesting research avenues for cherry intake within the context of chronic and acute intestinal

644 inflammation using conventional and germ-free mice to determine whether the cherry-induced
645 intestinal bacteria modulation could be beneficial in ameliorating or preventing the symptoms of
646 intestinal inflammation.
647
648 This study has drawbacks that ought to be taken into account in future studies. First, cherries
649 contain fiber and a wide variety of bioactive compounds and this is important because not all
650 compounds have the same properties on health. In this regard it was not the objective of this
651 study to identify each compound individually but to assess the effect of the fruit as a whole, at
652 least in a powder form. Second, in this study we used a model of genetic obesity but diet-induced
653 obesity can also shed light into the mechanisms associated with any health effect (*Hariri and*
654 *Thibault, 2010*). One main disadvantage of using diet-induced obesity is the high number of diets
655 that can be used to promote obesity and perhaps more importantly the nutritional differences and
656 outcomes in host physiology among these diets. Also, we know now that obesity in humans is a
657 complex disorder that often involves a genetic difference in the host. Importantly, Song *et al*.
658 (2016) showed a beneficial effect of cherries using a diet-induced model of obesity, thus
659 suggesting that cherries can have a positive impact in different types of obesity-related disorders.
660
661 **Conclusions**
662 In summary, this study shows that cherry supplementation for 12 weeks can modify the colon
663 microbiota and the concentrations of SCFAs. Our research model did not provide strong
664 evidence to suggest that this dietary intervention can lead to changes in biomarkers of
665 inflammation, cellular stress, and gut barrier function in colonic mucosal cells and colon tissues.
666 The reason of why the change in the microbiota and SCFAs did not affect the host physiology

remains to be investigated, but may be related to the obese genetic animal model used instead of using high fat, high sugar to induce obesity, which is known to stimulate intestinal inflammation (*Rahman et al., 2016*). Also, it has been shown that mice deficient for intestinal gluconeogenesis do not show the same metabolic benefits on body weight and glucose control induced by SCFAs, despite similar modifications in gut microbiota composition (*De Vadder et al., 2014*). Indeed, this topic is worth exploring further, especially in a context of diabetes (*Mithieux et al., 2004*). More research is desirable into the implications of cherry consumption as a dietary supplement in diabetic and obese human patients.

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Figure 1

Bar plots showing relative abundance (percentages, x axis) of the most abundant bacterial taxa at the family level.

A=Lean, B=Obese, C=Cherry supplemented group. Please note the noticeable difference in the abundance of the S24-7 group, Bacteroidaceae, Lactobacillaceae, *Akkermansia* (family Verrucomicrobiaceae), and Enterobacteriaceae (highlighted for better visualization). Statistical significant differences were found for these groups using either 16S sequencing, qPCR analyses, or both (see main text for details).

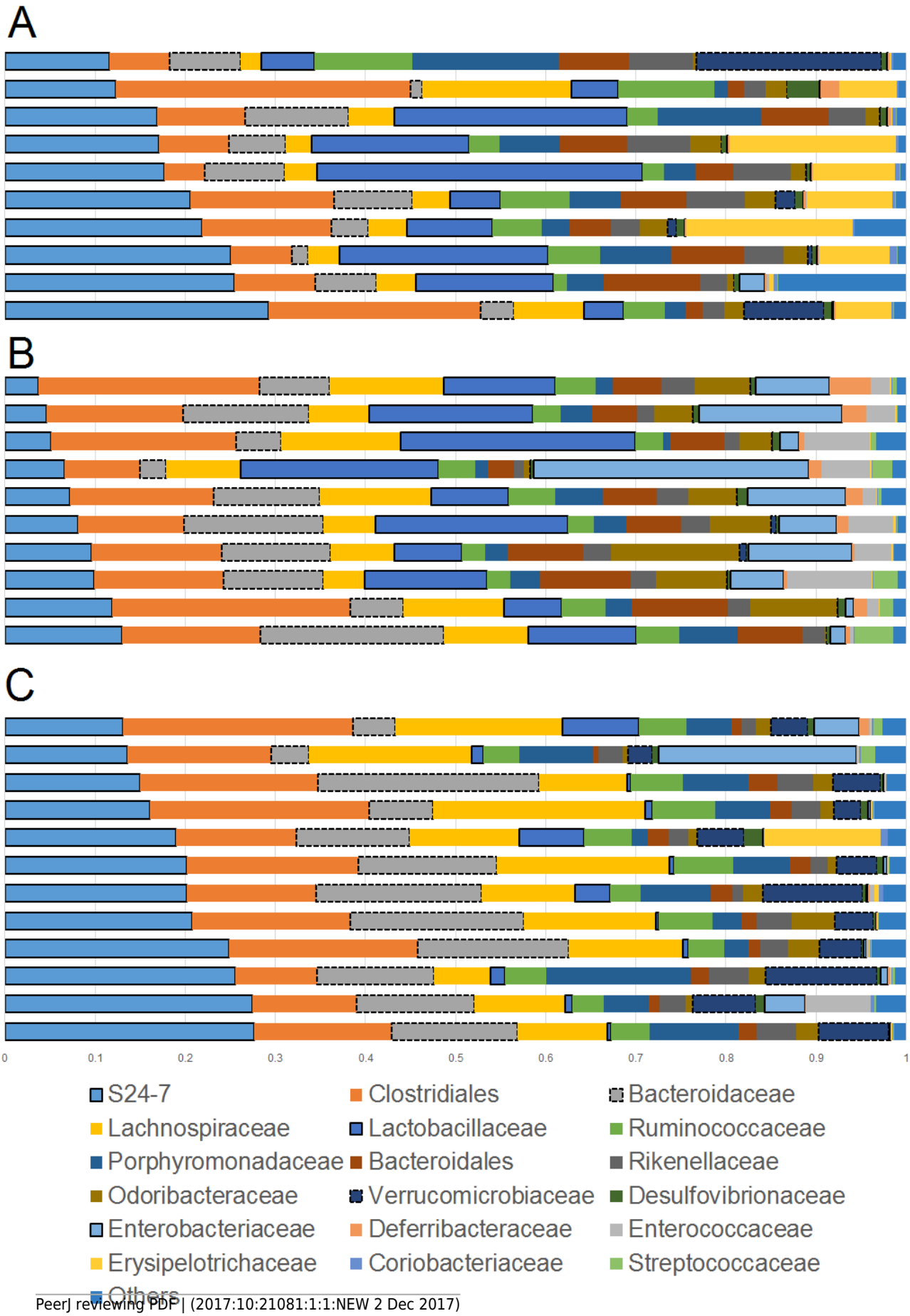
A
B
C
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
S24-7
Clostridiales
Bacteroidaceae
Lachnospiraceae
Lactobacillaceae
Ruminococcaceae
Porphyromonadaceae
Bacteroidales
Rikenellaceae
Odoribacteraceae
Verrucomicrobiaceae
Desulfovibrionaceae
Enterobacteriaceae
Deferribacteraceae
Enterococcaceae
Erysipelotrichaceae
Coriobacteriaceae
Streptococcaceae
Others

Figure 2

Alpha rarefaction plot for all treatment groups.

A flat line would indicate that the analysis of more sequences would not be able to detect more species (OTUs at 97% similarity).

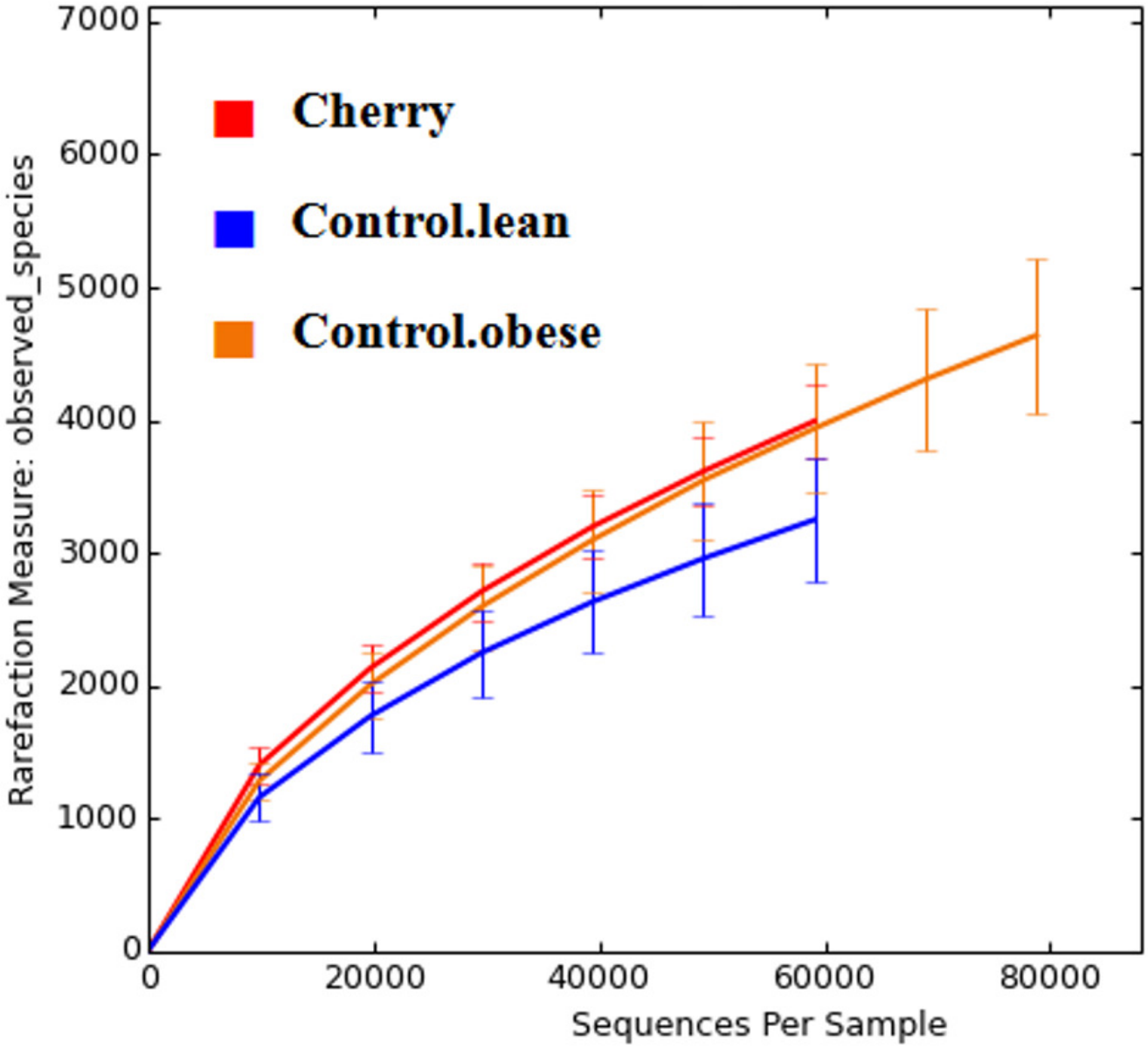

Figure 3

PCoA plots of weighted (A) and unweighted (B) UniFrac distance matrices.

Please note that the clustering of samples according to treatment is stronger (i.e. show less overlap) in the plot using the unweighted UniFrac distance matrix (p=0.001, R=0.844, ANOSIM test) compared to the plot using the weighted UniFrac distance matrix (p=0.001, R=0.716, ANOSIM test).

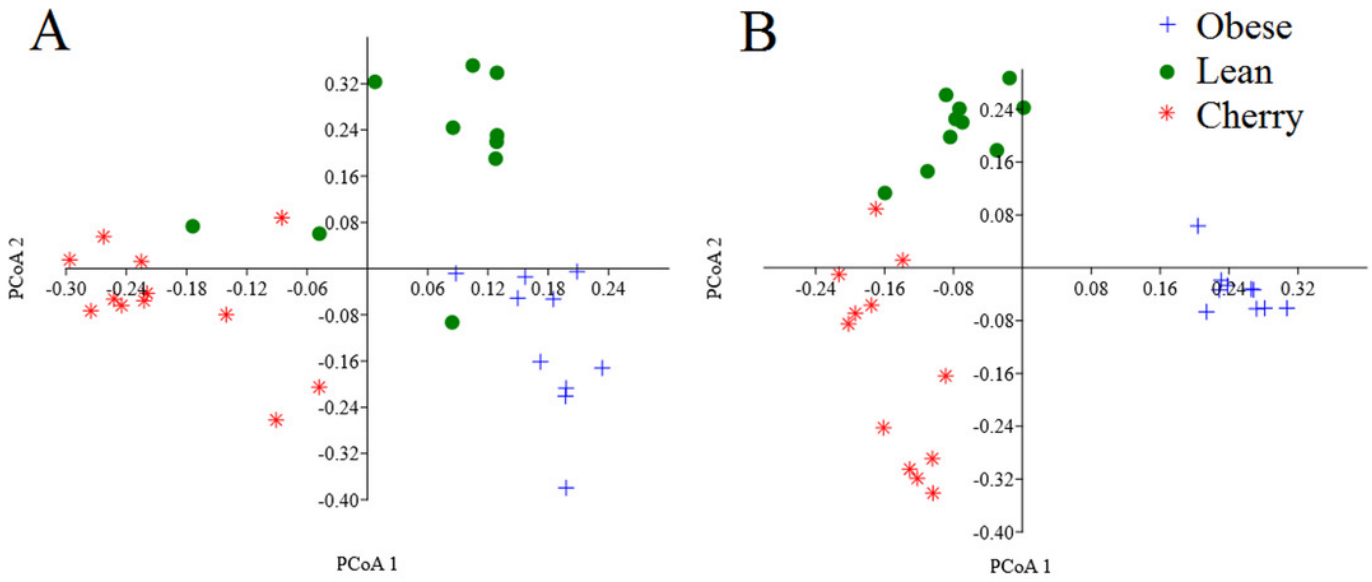

Figure 4

Boxplots showing qPCR results for selected bacterial groups in colon contents (or colon mucosa) that showed or almost reached statistical significance difference.

P values come from the Kruskal-Wallis test. A= *Akkermansia*, B= *Bacteroides fragilis*, C= *Bacteroides vulgatus*, D= *Bacteroides*/*Prevotella*, E= Bacteroidetes, E*=Bacteroidetes (colonic mucosa), F= Betaproteobacteria, F*=Betaproteobacteria (colonic mucosa), G=*Clostridium butyricum*, G*= *Clostridium butyricum* (colonic mucosa), H=*Clostridium* cluster IV, I= *E. coli*, J=Enterobacteriaceae, J*= Enterobacteriaceae (colonic mucosa), K= *Enterococcus*, L= *Faecalibacterium*, M=*Lactobacillus plantarum*, N=*Lactobacillus*, O= Ruminococcaceae, P=Tenericutes. qPCR data is expressed as log amount of amplified DNA (in picograms) per 10 ng of total isolated DNA.

A
p<0.0001
Obese Cherry Lean
B
p=0.0005
Obese Cherry Lean
C
p=0.0004
Obese Cherry Lean
D
p=0.0562
Obese Cherry Lean
E
p=0.0023
Obese Cherry Lean
E*
p=0.002
Obese Cherry Lean
F
p<0.0001
Obese Cherry Lean
F*
p<0.0001
Obese Cherry Lean
G
p<0.0001
Obese Cherry Lean
G*
p<0.0001
Obese Cherry Lean
H
p=0.0079
Obese Cherry Lean
I
p<0.0001
Obese Cherry Lean
J
p<0.0001
Obese Cherry Lean
J*
p<0.0001
Obese Cherry Lean
K
p<0.0001
Obese Cherry Lean
L
p=0.0022
Obese Cherry Lean
M
p=0.0003
Obese Cherry Lean
N
p<0.0001
Obese Cherry Lean
O
p=0.0292
Obese Cherry Lean
P
p<0.0001
Obese Cherry Lean

Table 1(on next page)

Diets utilized in this study without cherry supplementation (control) and with 10% cherry supplementation.

[1]AIN-93G-MX supplied by Dyets Inc. (Bethlehem, PA), containing (g/kg): Calcium Carbonate (357), Potassium Phosphate, monobasic (196), Potassium Citrate .H20 (70.78), Sodium Chloride (74), Potassium Sulfate (46.6), Magnesium Oxide (24), Ferric Citrate, U.S.P. (6.06), Zinc Carbonate (1.65), Manganous Carbonate (0.63), Cupric Carbonate (0.3), Potassium Iodate (0.01), Sodium Selenate (0.01025), Ammonium Paramolybdate .4H20 (0.00795), Sodium Metasilicate .9H20 (1.45), Chromium Potassium Sulfate .12H20 (0.275), Lithium Chloride (0.0174), Boric Acid (0.0815), Sodium Fluoride (0.0635), Nickel Carbonate (0.0318), Ammonium Vanadate (0.0066), Sucrose, finely powdered (221.026). [2]AIN-93G Vitamin Mix supplied by Dyets Inc. (Bethlehem, PA), containing (g/kg): Niacin (3), Calcium Pantothenate (1.6), Pyridoxine HCl (0.7), Thiamine HCl (0.6), Riboflavin (0.6), Folic Acid (0.2), Biotin (0.02), Vitamin E Acetate (500 IU/g) (15), Vitamin B12 (0.1%) (2.5), Vitamin A Palmitate (500 000 IU/g) (0.8), Vitamin D3 (400 000 IU/g) (0.25), Vitamin K1/Dextrose Mix (10 mg/g) (7.5), Sucrose (967.23). [3]Cherry powder contributed with 5.1 g fiber/kg diet and 759 mg GAE/100 g of total phenolics (629 mg GAE/100g extractable and 130 mg GAE/100g non-extractable or bound phenolics). Cherry powder was processed by Powder Pure (The Dalles, OR) and contains 80% dark sweet cherry puree (Bing variety), 20% organic rice maltodextrin and 2% silicon dioxide.

Table 1 Diets utilized in this study without cherry supplementation (control) and with 10% cherry supplementation.

	Control		**Cherry 10%**	
Ingredient	Weight (g)	Kcal	Weight (g)	Kcal
Casein	100	400	100	400
Maltodextrin	66	264	66	264
Sucrose	50	200	50	200
Cellulose	25	0	25	0
Mineral Mix[1]	17.5	0	17.5	0
Vitamin Mix[2]	5	0	5	0
L-Cysteine	1.5	6	1.5	6
Choline Bitartrate	1.25	0	1.25	0
t-Butylhydroquinone	0.007	0	0.007	0
Cornstarch	198.75	795	98.75	395
Soybean Oil	35	315	35	315
Cherry powder[3]	0	0	100	400
Agar	*20*	*0*	*20*	*0*
Water	*480*	*0*	*480*	*0*
Total	**1,000**	**1,980**	**1,000**	**1,980**

[1]AIN-93G-MX supplied by Dyets Inc. (Bethlehem, PA), containing (g/kg): Calcium Carbonate (357), Potassium Phosphate, monobasic (196), Potassium Citrate .H20 (70.78), Sodium Chloride (74), Potassium Sulfate (46.6), Magnesium Oxide (24), Ferric Citrate, U.S.P. (6.06), Zinc Carbonate (1.65), Manganous Carbonate (0.63), Cupric Carbonate (0.3), Potassium Iodate (0.01), Sodium Selenate (0.01025), Ammonium Paramolybdate .4H20 (0.00795), Sodium Metasilicate .9H20 (1.45), Chromium Potassium Sulfate .12H20 (0.275), Lithium Chloride (0.0174), Boric Acid (0.0815), Sodium Fluoride (0.0635), Nickel Carbonate (0.0318), Ammonium Vanadate (0.0066), Sucrose, finely powdered (221.026). [2]AIN-93G Vitamin Mix supplied by Dyets Inc. (Bethlehem, PA), containing (g/kg): Niacin (3), Calcium Pantothenate (1.6), Pyridoxine HCl (0.7), Thiamine HCl (0.6), Riboflavin (0.6), Folic Acid (0.2), Biotin (0.02), Vitamin E Acetate (500 IU/g) (15), Vitamin B12 (0.1%) (2.5), Vitamin A Palmitate (500 000 IU/g) (0.8), Vitamin D3 (400 000 IU/g) (0.25), Vitamin K1/Dextrose Mix (10 mg/g) (7.5), Sucrose (967.23).

[3]Cherry powder contributed with 5.1 g fiber/kg diet and 759 mg GAE/100 g of total phenolics (629 mg GAE/100g extractable and 130 mg GAE/100g non-extractable or bound phenolics). Cherry powder was processed by Powder Pure (The Dalles, OR) and contains 80% dark sweet cherry puree (Bing variety), 20% organic rice maltodextrin and 2% silicon dioxide.

Table 2(on next page)

PICRUSt results (average percentages) for the statistically significant features ($p<0.05$ adjusted p-values*).

↓ lowest, ↑ highest. *We removed 5 features related to human diseases that also reached statistical significance because of their questionable relevance to this study.

1 **Table 2** PICRUSt results (average percentages) for the statistically significant features ($p<0.05$
2 adjusted p-values*).
3

Level 1	Level 2	Level 3	Obese controls	Obese supplemented	Lean controls	Adjusted p values
Metabolism	Amino Acid Metabolism	Amino acid related enzymes	1.56↓	1.64	1.74↑	0.008
Metabolism	Amino Acid Metabolism	Histidine metabolism	0.61↓	0.72	0.79↑	0.011
Metabolism	Biosynthesis of Other Secondary Metabolites	Stilbenoid, diarylheptanoid and gingerol biosynthesis	0.00↓	0.00	0.01↑	0.020
Metabolism	Carbohydrate Metabolism	Ascorbate and aldarate metabolism	0.21↑	0.19	0.13↓	0.023
Metabolism	Carbohydrate Metabolism	Butanoate metabolism	0.85↑	0.74	0.73↓	0.038
Metabolism	Carbohydrate Metabolism	Pentose and glucuronate interconversions	0.70	0.73↑	0.62↓	0.049
Metabolism	Carbohydrate Metabolism	Pentose phosphate pathway	0.98↓	1.09↑	1.01	0.033
Metabolism	Energy Metabolism	Carbon fixation in photosynthetic organisms	0.68↓	0.74↑	0.73	0.013
Metabolism	Enzyme Families	Peptidases	2.08↓	2.14	2.32↑	0.026
Metabolism	Enzyme Families	Protein kinases	0.49↑	0.40	0.34↓	0.004
Metabolism	Glycan Biosynthesis and Metabolism	Peptidoglycan biosynthesis	0.83↓	0.84	0.94↑	0.039
Metabolism	Lipid Metabolism	alpha-Linolenic acid metabolism	0.03↑	0.01	0.01↓	0.025
Metabolism	Metabolism of Cofactors and Vitamins	One carbon pool by folate	0.64↓	0.72	0.76↑	0.036
Metabolism	Metabolism of Cofactors and Vitamins	Thiamine metabolism	0.51↓	0.56	0.58↑	0.035
Metabolism	Metabolism of Terpenoids and Polyketides	Terpenoid backbone biosynthesis	0.54↓	0.61	0.69↑	0.003
Metabolism	Xenobiotics Biodegradation and Metabolism	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) degradation	0.00↓	0.00↑	0.00	0.015
Genetic Information Processing	Replication and Repair	Mismatch repair	0.84↓	0.92	0.97↑	0.005
Genetic Information Processing	Translation	Ribosome	2.26↓	2.46	2.82↑	0.006
Genetic Information Processing	Replication and Repair	DNA replication proteins	1.28↓	1.38	1.49↑	0.006
Genetic Information	Translation	Translation factors	0.54↓	0.59	0.66↑	0.006

Processing						
Genetic Information Processing	Replication and Repair	Base excision repair	0.47↓	0.51	0.55↑	0.011
Genetic Information Processing	Replication and Repair	DNA repair and recombination proteins	2.99↓	3.15	3.37↑	0.012
Genetic Information Processing	Replication and Repair	Nucleotide excision repair	0.37↓	0.45	0.46↑	0.014
Genetic Information Processing	Replication and Repair	DNA replication	0.69↓	0.74	0.82↑	0.016
Genetic Information Processing	Translation	Aminoacyl-tRNA biosynthesis	1.16↓	1.25	1.39↑	0.016
Genetic Information Processing	Folding, Sorting and Degradation	Protein export	0.61↓	0.65	0.72↑	0.017
Genetic Information Processing	Transcription	RNA polymerase	0.16↓	0.17	0.20↑	0.019
Genetic Information Processing	Replication and Repair	Homologous recombination	0.95↓	0.99	1.10↑	0.022
Environmental Information Processing	Signal Transduction	Two-component system	2.30↑	1.91	1.63↓	0.024
Environmental Information Processing	Signaling Molecules and Interaction	Bacterial toxins	0.12↓	0.16	0.16↑	0.025

↓ lowest, ↑ highest. *We removed 5 features related to human diseases that also reached statistical significance because of their questionable relevance to this study.

Table 3(on next page)

Median (minimum-maximum) SCFA concentrations (µmol/mg caecal contents).

P values come from either the Kruskal-Wallis test or the Mann Whitney test when comparing only two treatment groups due to lack of detectable values in one group. Different letters state statistical significance difference. The symbol (-) is included to denote treatment groups where all or most samples were undetectable. The number of samples (n) in which the specific SCFA was detected for each experimental group is also included. For most SCFAs, we chose not to perform a statistical comparison because of very low sample size in at least one treatment group (NA or not applicable). Please note that most samples (especially from lean and obese controls) showed undetectable levels of several SCFAs. In our experience this was not due to errors in our analytical methodology, and this is supported by the fact that all samples were treated equally yet most samples from supplemented mice did show detectable levels of most SCFAs.

Table 3 Median (minimum-maximum) SCFA concentrations (μmol/mg caecal contents).

	Obese controls	Obese supplemented	Lean controls	*P* value
Caproate	1.2(0.4-3.9)[a] (n=9)	285(217-437)[b] (n=12)	1.0(0.4-652)[a] (n=10)	0.0033
Methyl butyrate	-	116(17-405) (n=12)	62(43-92) (n=3)	NA
Butyrate	6.2(5.3-20)[a] (n=9)	-	11.9(6.1-16.2)[a] (n=7)	0.3511
Propionate	-	384(258-649) (n=12)	356(281-438) (n=4)	NA
Acetate	1.9(1.4-1.9) (n=3)	269.4(128-672) (n=12)	273.2(40-351) (n=3)	NA
Valerate	-	15.4(4-48) (n=10)	-	NA

P values come from either the Kruskal-Wallis test or the Mann Whitney test when comparing only two treatment groups due to lack of detectable values in one group. Different letters state statistical significance difference. The symbol (-) is included to denote treatment groups where all or most samples were undetectable. The number of samples (n) in which the specific SCFA was detected for each experimental group is also included. For most SCFAs, we chose not to perform a statistical comparison because of very low sample size in at least one treatment group (NA or not applicable). Please note that most samples (especially from lean and obese controls) showed undetectable levels of several SCFAs. In our experience this was not due to errors in our analytical methodology, and this is supported by the fact that all samples were treated equally yet most samples from supplemented mice did show detectable levels of most SCFAs.