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ORIGINAL CONTRIBUTION

Effect of barley supplementation on the fecal microbiota, caecal biochemistry, and key biomarkers of obesity and inflammation in obese db/db mice

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Abstract
Purpose Barley is a low-glycemic index grain that can help diabetic and obese patients. The effect of barley intake depends on the host and the associated gut microbiota. This study investigated the effect of barley intake on the fecal microbiota, caecal biochemistry, and key biomarkers of obesity and inflammation.
Methods Obese db/db mice were fed diets with and without barley during 8 weeks; lean mice were used as lean controls. Fecal microbiota was evaluated using 16S marker gene sequencing in a MiSeq instrument; several markers of caecal biochemistry, obesity, and inflammation were also evaluated using standard techniques.
Results Bacterial richness (i.e., Operational Taxonomic Units) and Shannon diversity indexes were similar in all obese mice (with and without barley) and higher compared to lean controls. Barley intake was associated with increased abundances of *Prevotella, Lactobacillus*, and the fiber-degraders S24-7 (Candidatus Homeothermaceae) compared to both lean and obese controls. The analysis of unweighted UniFrac distances showed a separate clustering of samples for each experimental group, suggesting that consumption of barley contributed to a phylogenetically unique microbiota distinct from both obese and lean controls. Caecal butyrate concentrations were similar in all obese mice, while succinic acid was lower in the barley group compared to obese controls. Barley intake was also associated with lower plasma insulin and resistin levels compared to obese controls.
Conclusions This study shows that barley intake is associated with a different fecal microbiota, caecal biochemistry, and obesity biomarkers in db/db mice that tend to be more similar to lean controls.

Keywords Obesity · Diabetes · Barley · Microbiota · 16S rRNA gene · Short-chain fatty acids

Introduction

Mammals and other animals are made of tissues that have evolved in the presence of trillions of different microorganisms over vast periods of time, a phenomenon that has shaped the characteristics of both forms of life [1]. This microbiota mostly inhabits the mucosal surfaces that are in contact with the exterior world such as the respiratory, reproductive, and digestive systems as well as the skin. The metabolic activity of the microbiota, especially the gut microbiota, is crucial to health but the normal microbe–host homeostasis can be disrupted during the course of disease (e.g., obesity, Inflammatory Bowel Disease) in a complex phenomenon known as microbial dysbiosis [2].

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Obesity is a growing health concern that has promoted collaborative interdisciplinary efforts to study the disease in order to help patients ameliorate the clinical signs. Several alternatives do exist for helping patients with obesity-related disorders (e.g., exercise, surgical procedures) but dietary manipulation is an easy and feasible strategy to help relieve symptoms and improve quality of life in these patients [3]. While dietary changes alone are not necessarily a remedy for everyone in all circumstances [4], accumulating evidence shows a potential of dietary interventions to influence health in obese and overweighed patients [5]. Research in this area is valuable for the millions of patients worldwide with body weight disorders and related health problems in both developed and developing countries [6].

Several research groups have examined the efficacy of dietary supplements to prevent and/or modify body weight and metabolism. Examples include prebiotics and probiotics [7] and anti-oxidants such as polyphenols [3, 8]. Since the gut microbiota is closely involved in the digestion and absorption of nutrients, dietary disturbances consequently lead to responses of gut microbial communities [9] that have a direct impact on the immune system [10]. Recent advances in these areas have allowed the emergence of key findings about the behavior of the gut microbial ecosystem under different dietary regimens [11].

Barley is an interesting food that has one of the lowest glycemic indexes, meaning it is one of the food ingredients with the lowest effect of blood glucose levels, while at the same time providing nutrients such as fiber, protein, vitamins, and minerals [12]. Several mechanisms have been proposed to explain the physiological effects of barley, including a delay of intestinal absorption of glucose and lipids and increased excretion of bile acids [13, 14]. Interestingly, barley has also been shown to exert an effect on the gut microbiota in humans and various animal species [15, 16], thus suggesting a potential link between the health benefits associated with consumption of barley and the gut microbial ecosystem. However, additional studies are needed to investigate this phenomenon more deeply especially in a context of obesity-related disorders. Therefore, the objective of this research was to investigate the effect of barley supplementation on fecal microbiota structure, caecal biochemistry, and key obesity biomarkers using an in vivo model of obesity.

Materials and methods

Ethical approval

This study has been approved by the Institutional Animal Care and Use Committee at Washington State University (WSU, IACUC# 04436-001).

Experimental design

Mice (all male, 4–5 weeks of age) were located in a controlled constant environment, visually inspected every day, and received food and water ad libitum throughout the whole study period. Leptin receptor-deficient obese db/db mice (BKS.Cg-+Lepr^{db}/+Lepr^{db}/OlaHsd—fat, black, homozygous) were used as the in vivo obesity model due to its usefulness to study obesity-associated disorders [17]. After 1 week of acclimatization, obese db/db mice were randomly assigned to obese-control group (n = 10) fed with AIN-93G standard diet or barley group (n = 10) fed with a modified AIN-93G diet containing barley as a source of carbohydrates and proteins (barley group, Table 1). Control (i.e., AIN-93G) and experimental diets were offered for a period of 8 weeks. The AIN-93G diet was also used during acclimatization. The approach to formulate the barley diet was to normalize as much as possible carbohydrates, proteins, and caloric density to those contents in the standard diet (AIN-93G) using barley as a source of these nutrients (Table 1). However, both diets (with and without barley supplementation) did not have the exact amount of protein and carbohydrates (Table 1) and such small differences may be sufficient to exert an effect on gut physiology and microbial activity. The BKS.Cg-Dock^{7m}+/+Lepr^{db}/OlaHsd—lean, black, heterozygous mice were used as lean controls (n = 11) for the obese db/db control because they have the closely linked recombinant genes Dock^{7m}+/+Lepr^{db} instead of the +Lepr^{db}/+Lepr^{db} that makes the obese counterparts leptin receptor deficient. Lean controls were fed the standard AIN-93G diet used to feed obese-control mice (Table 1). All mice were purchased from Harlan Laboratories (Kent, WA, USA) and diets were formulated and prepared by Dyets Inc. (Bethlehem, PA, USA), shipped overnight to WSU and maintained at 4 °C for use in animal feeding. In this study, we used the barley variety 'Havener' (two-row, hulless, spring food barley variety with the following seed characteristics: protein 13.8%, moisture 11%, β-glucan 6%). Please note that up to 10% of barley β-glucans was well tolerated by Wistar rats without any signs of toxicity [18]. Barley was conventionally grown at Spillman Farm in Pullman, WA, USA. Compared to the more common hulled barley types, which require a pearling process to remove the hull, hulless barley is the whole grain form of barley, in which the hull naturally separates during harvest. Barley grains were ground with a cyclone mill (Udy, Boulder, CO, USA) through 0.5-mm sieving rings to obtain barley flour which was used for diet preparation.

Body weight was recorded once a week and food consumption was recorded every other day. Two mice from the barley-supplemented group died during the last week of the study for unknown reasons (from these animals we were only able to collect feces for analysis of microbiota). At the end of the study (8 weeks), mice were fasted overnight,

Table 1 Formulation of experimental diets (g/kg)

Ingredients	Lean and obese diet	Barley diet
Casein, high nitrogen	200	0.0
L-Cysteine	3	3
Sucrose	100	0.0
Cornstarch	397.486	0.0
Dyetrose	132	0.0
Soybean oil	70	70
t-Butylhydroquinone	0.014	0.014
Cellulose	50	0.0
Mineral mix #210025	35	35
Vitamin mix #310025	10	10
Choline bitartrate	2.5	2.5
Barley (13.8% protein, 6% β-glucan, 11% moisture, 2.3% lipids, 17.3% total dietary fiber, 73% total carbohydrates by difference)	0	879.5
Total (g)	1000	1000
kcal/kg	3760.0	3591.0

Modified AIN-93G Purified Rodent Diet with barley prepared by Dyets Inc. (Bethlehem, PA, USA)

exposed to inhalation with CO_2 overdose until the animal become unconscious, blood was obtained by cardiac puncture, and animals were terminated by cervical dislocation. Blood (~100 to ~500 µL) was collected from $n = 8$ (barley group), $n = 10$ (obese control), and $n = 11$ (lean control), transferred into a tube containing 10 µL of ethylenediaminetetraacetic acid (EDTA) (Sommerville, NJ, USA), and centrifuged at 5000*g* at 4 °C for 10 min to separate plasma from erythrocytes. The erythrocytes were stored at −80 °C for reactive oxygen species (ROS) determination, and plasma was aliquoted and stored at −80 °C for future use. The liver, heart, kidney, subcutaneous fat, epididymal fat, heart fat, and abdominal fat were dissected and weighed. Liver tissues were flash-frozen in liquid nitrogen, and stored at −80 °C for future analysis. The weight of the cecum and its contents were also recorded for analysis.

Body mass index and adiposity index

Body mass indexes (BMI) were determined by dividing body weight (kg) by body length (m^2) [19]. Adiposity index was determined by the sum of weights of abdominal, epididymal, heart, and subcutaneous fat divided by body weight × 100 and expressed as adiposity index [19]. Caloric intake was calculated by multiplying the food intake (g) by caloric density (calories/g) in each diet (Table 1).

Fecal collection and DNA extraction

Fecal samples were collected from terminal colon and rectum in the moment of euthanasia at the end of the study (8 weeks). Total genomic DNA was extracted and purified using bead-beating followed by a commercial DNA extraction kit (QIAamp DNA Stool Mini Kit, Qiagen Inc., Valencia, CA, USA) as shown elsewhere [20, 21]. The concentration and purity of all DNA samples was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR and high-throughput sequencing of 16S rRNA genes

The 16S rRNA gene contains both conserved and variable regions that allow for differentiation of bacterial groups. We used primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) that can amplify the V4 semi-conserved region (~250 bp) as described elsewhere [22]. Sequencing was performed using an Illumina MiSeq instrument at the Genome Center DNA Technologies Core at the University of California Davis as described previously [20, 21]. All raw 16S sequences are available at the Sequence Read Archive (SRA, NCBI) under accession numbers: PRJNA281761 and PRJNA314690. The trim.seqs command in MOTHUR [23] was utilized for splitting raw fastq files for uploading data to SRA.

Bioinformatics

Raw fastq files were demultiplexed (i.e., assignment of reads to samples), quality filtered, and analyzed using default scripts and parameters in Quantitative Insights into Microbial Ecology (QIIME) v. 1.8.0 [24, 25]. Operational Taxonomic Units (OTUs) were assigned using two approaches. First, using the open-reference algorithm described by Rideout et al. [26] for alpha and beta diversity analyses. This open approach is very useful at assigning sequences

to groups because it does not necessarily discard sequence data when there is no match with databases. UCLUST [27] was used to classify sequences into groups and also to assign taxonomy (this is the default method in the pick_open_reference_otus.py QIIME script). Second, using a closed reference algorithm as currently required by PICRUSt (see Prediction of metabolic profiles below). The Greengenes 13_5 97% OTU representative 16S rRNA gene sequences was used as the reference sequence collection [28].

Prediction of metabolic profiles

The Galaxy platform of Phylogenetic Investigation of Communities by Reconstruction of Unobserved states (PICRUSt, [29]) was used to predict the functional profile of the microbial communities using the OTU table from the closed reference algorithm described above. The current galaxy version of PICRUSt supports several types of functional predictions; in this study, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs [30].

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

Fresh distal caecal contents were collected from all mice at the end of the study (8 weeks) and SCFAs were quantified using a High-Performance Liquid Chromatography(HPLC)-Photo-diode array (PDA) analysis system as reported elsewhere [20, 21, 31].

Obesity biomarkers in plasma

Analysis of plasma was performed in a subset of samples ($n = 4$ to $n = 11$) due to limitations in collected blood volumes; therefore, each specific analysis was carried out in a different subset of samples depending on several factors including the relevance of each analysis for the study and the amount of material (plasma in µL) needed to perform the analysis. Plasma glucose, triglycerides (TG), high-density lipoprotein cholesterol (HDL-c), and total cholesterol concentrations (Total-c) were measured using commercial kits according to manufacturer's protocol (Wako Diagnostics, Richmond, VA, USA). Low-density lipoprotein (LDL-c) and very-low-density lipoprotein (VLDL-c) were determined according to [32] (LDL-c = Total-c − HDL-c − TG/5; VLDL-c = TG/5). Plasma levels of lipid peroxidation were measured using the thiobarbituric acid reactive substance (TBARS) assay (Cayman Chemical Co. Ann Arbor, MI, USA), according to the manufacturer's protocol. The level of lipid peroxidation was expressed as µM malondialdehyde (MDA). Protein carbonyls in plasma were determined using the 2,4-dinitrophenylhydrazine (DNPH) reagent as reported previously [33] and adapted to microplate. Briefly, 25 µL of plasma were mixed with 25 µL 10 mM of DNPH in 2.5 M HCl, incubated at room temperature, and vortexed every 5 min. Protein carbonyls were precipitated with 12.5 µL of 10% w/v trichloroacetic acid (TCA) on ice-bath for 30 min followed by centrifugation at 9000*g* for 15 min (4 °C). Pellets were washed three times with cold ethanol/ethyl acetate solution (1:1 v/v) to eliminate the excess of DNPH and dissolved in 100 µL of 6 M guanidine-HCl. Absorbance was measured at 370 nm against a blank without DNPH. Protein carbonyls were determined using extinction coefficient of DNPH (ε = 22,000 mol/L/cm) and expressed as nmol/mg protein quantified by Bradford assay (Bio-Rad, Hercules, CA, USA). Plasma levels of interleukin (IL)-6, plasminogen activator inhibitor-1 (PAI-1), leptin, insulin, and resistin were quantified with the multiplex magnetic bead-based immunoassay Luminex system using the Milliplex mouse adipokine kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. Data were analyzed using Luminex xPonent 3.0 software (Austin, TX, USA).

ROS in erythrocytes

Blood erythrocytes were analyzed for production of ROS using a fluorometric assay that is based on the oxidation of the fluorochrome, 2′,7′-dichlorofluoroscein-diacetate (DCFH-DA; Sigma-Aldrich, St Louis, MO, USA) as previously reported [34]. Briefly, 900 µL of phosphate-buffered saline (PBS), pH 7.4 was used to wash the erythrocytes (100 µL), followed by centrifugation at 1500*g* for 5 min. The supernatant was discarded and condensed erythrocytes were diluted with PBS to a final content of 5% erythrocytes. Aliquots of 50 µL of erythrocyte suspension were added to a 96-well plate followed by 50 µL of 100 mM of DCFH-DA. The rate at which DCFH-DA was oxidized by intracellular ROS to DCF was determined fluorometrically. Fluorescence at 484 nm extinction and 535 nm emission was determined after 60-min reaction. Results were expressed in arbitrary relative fluorescence units (RFU).

Liver cholesterol

Liver cholesterol was extracted as previously reported [35] with minor modifications. Briefly, frozen liver samples were weighed and ground with liquid nitrogen into a homogenous powder in a mortar. Approximately 100 mg of ground tissue was added to 2 mL of chloroform/methanol (2:1 v/v) solution and homogenized in high speed homogenizer (Tekmar TR-10, Vernon, BC, Canada) for 2 min at 60% output. The mixture was centrifuged at 3000 rpm at 4 °C for 20 min. Supernatants were collected into 500-µL Eppendorf tube and mixed with 100 µL deionized water followed by centrifugation for 30 s at 3000 rpm at room temperature. The supernatant (methanol-water) was discarded and the chloroform

phase was evaporated in a vacuum evaporator. The dry residue was resuspended in 100 µL isopropanol to measure total cholesterol using a commercial kit (Wako Diagnostics, Richmond, VA, USA). Liver weight values were used to calculate total liver cholesterol levels (mg cholesterol/liver).

Statistical analysis

Unless otherwise noted, the non-parametric alternative Kruskal–Wallis test was used to compare our dependent variables (e.g., final body weights, obesity biomarkers) in PAST [36]. Multiple comparisons were performed using Mann–Whitney tests and adjustment for multiple comparisons was performed using the Bonferroni method. In an effort to investigate possible differences in body weight gain over time, the MIXED procedure in SAS University Edition was used to analyze the dependent variable body weight with the independent variables "treatment" as a discrete fixed effect and "week" as a continuous explanatory covariate in a mixed model. This additional statistical analysis is very important because dietary ingredients may not be necessarily related with differences in final body weights (i.e., at the end of the study) but instead with delays in body weight gain over time, as recently shown by our research group [21]. An Analysis of Similarities (ANOSIM) and the Adonis test were used to test for clustering of microbial communities using both weighted and unweighted UniFrac distance matrices in QIIME. The difference between weighted and unweighted UniFrac is that the former uses information about the number of sequences as well as the phylogenetic information contained in the sequences, while the unweighted UniFrac does not take into account the number of sequences. Using both metrics is very useful to inform about the factors that structure microbial communities [20, 21, 37, 38]. The Linear Discriminant Analysis (LDA) Effect Size (LEfSe) method was used to investigate differences in microbial communities using a LDA score threshold of three [39]. Principal Coordinate Analysis (PCoA) was performed in PAST using the weighted and unweighted distance matrices obtained from QIIME. STAMP [40] was used to analyze the PICRUSt data with ANOVA and False Discovery Rate. An alpha of 0.05 was used to reject null hypotheses.

Results

Physical and dietary parameters of mice

Body weight of all obese mice (with and without barley) was similar (median = 47.3 g for supplemented mice; median = 45.6 g for obese controls) at the end of the study (8 weeks) and both were significantly higher compared to lean mice (median = 30 g, $P < 0.0001$, Kruskal–Wallis test). While food consumption was higher in barley group (average 5.9 ± 0.87 g food/animal/day) compared to obese-control group (4.4 ± 0.4 g food/animal/day) and to lean control (3.0 ± 0.1 g food/animal/day) ($P < 0.001$, Kruskal–Wallis test), the body weight gain was similar between obese control and barley throughout the study period (Fig. 1). Interestingly, the BMI in barley group was higher compared to obese control and lean control but this phenomenon was not due to body fat accumulation since barley group and obese control had similar adiposity indexes (Supplementary Table S1). The higher BMIs in barley-supplemented mice (compared to obese controls) was due to a higher body weight (although not statistically significantly, $P = 0.3922$ at the end of the study, Mann–Whitney test) and a relatively lower body length (also not statistically significant when compared to obese controls, $P = 0.2166$, Mann–Whitney test), which may be related to the difference in caloric intake (Table 1; Table S1). Consumption of barley was associated with higher cecum weight and cecum content compared to obese-control group (Table S1). Liver, heart, and kidney weights were not different among treatment groups (Table S1).

Microbial membership composition

All quality-filtered reads were assigned to 5366 OTUs using an open OTU picking algorithm that does not discard sequences for not matching reference databases [26]. As shown in other studies, the fecal microbiota of all mice was comprised mostly by Firmicutes and Bacteroidetes (median >90% of both phyla together) (Fig. 2). Lean mice had the highest abundance of Firmicutes and the lowest Bacteroidetes, while barley-supplemented mice showed opposite abundances of these two major phyla: barley group had ~55% of Bacteroidetes compared to ~25% in lean mice and only ~40% of Firmicutes compared to ~65% in lean mice (Fig. 2). Interestingly, the microbiota associated with barley supplementation did not resemble the obese microbiota either (Fig. 2). The significance of these results for the abundant phyla Firmicutes and Bacteroidetes is at best modest due to the many—often divergent—bacterial groups that constitute each of these phyla at lower taxonomic levels. Other phyla showed low abundances including Actinobacteria, Proteobacteria, and others (Fig. 2).

The LEfSe method is useful at finding potentially relevant bacterial groups that are significantly enriched in each treatment group [39]. In this study, each treatment group was associated with higher abundances of distinctive subsets of taxa (Fig. 3; Supplementary Figure S1), thus confirming that barley helped output a unique microbial membership composition that is different from both lean and obese controls. For example, barley intake was associated with increased relative abundances

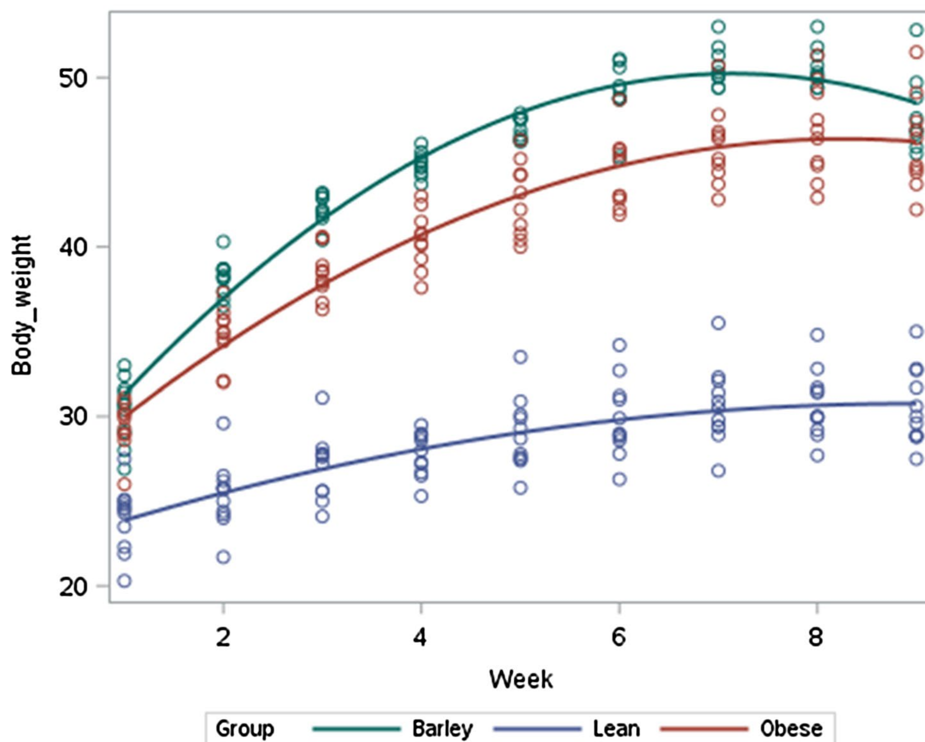

Fig. 1 Body weight (g) in lean and obese mice with (barley group) and without (obese-control group) barley supplementation. Despite a significant difference in slopes between the obese groups with and without barley, there was no statistically significant difference in body weight between the barley group and the obese-control group in all time points

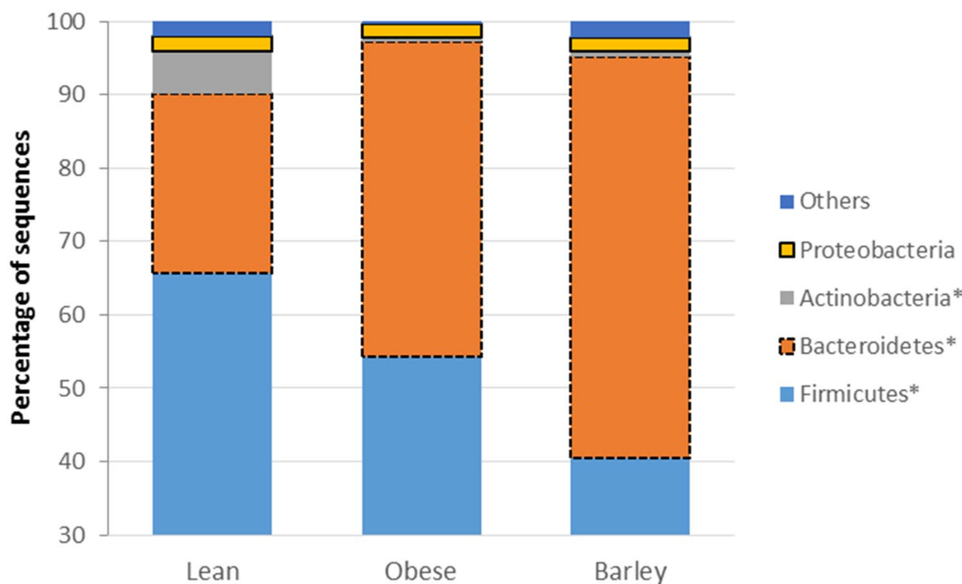

Fig. 2 Composition of fecal microbiota at the phylum level. Please note that the *y* axis (percentage of sequences) was modified to allow visualization of low abundant groups (e.g., Proteobacteria). Bacteroidetes and Proteobacteria are highlighted using different border styles for better visualization. *Asterisks* denote groups that were significantly different among treatment groups based on a Kruskal–Wallis test ($P < 0.05$)

of *Prevotella, Lactobacillus*, the fiber-degraders S24-7 (Candidatus Homeothermaceae) as well as Mogibacteriaceae (Fig. 3; Fig. S1). The finding that barley intake is associated with a distinctive microbiota is further supported by the separate clustering of bacterial relative abundances for each experimental group (Fig. 4).

Diversity analyses

Alpha and beta diversity (see below) were calculated using 1925 sequences per sample because this was the lowest number of sequences obtained in a given sample (minimum = 1925; maximum = 16,869 sequences). There was

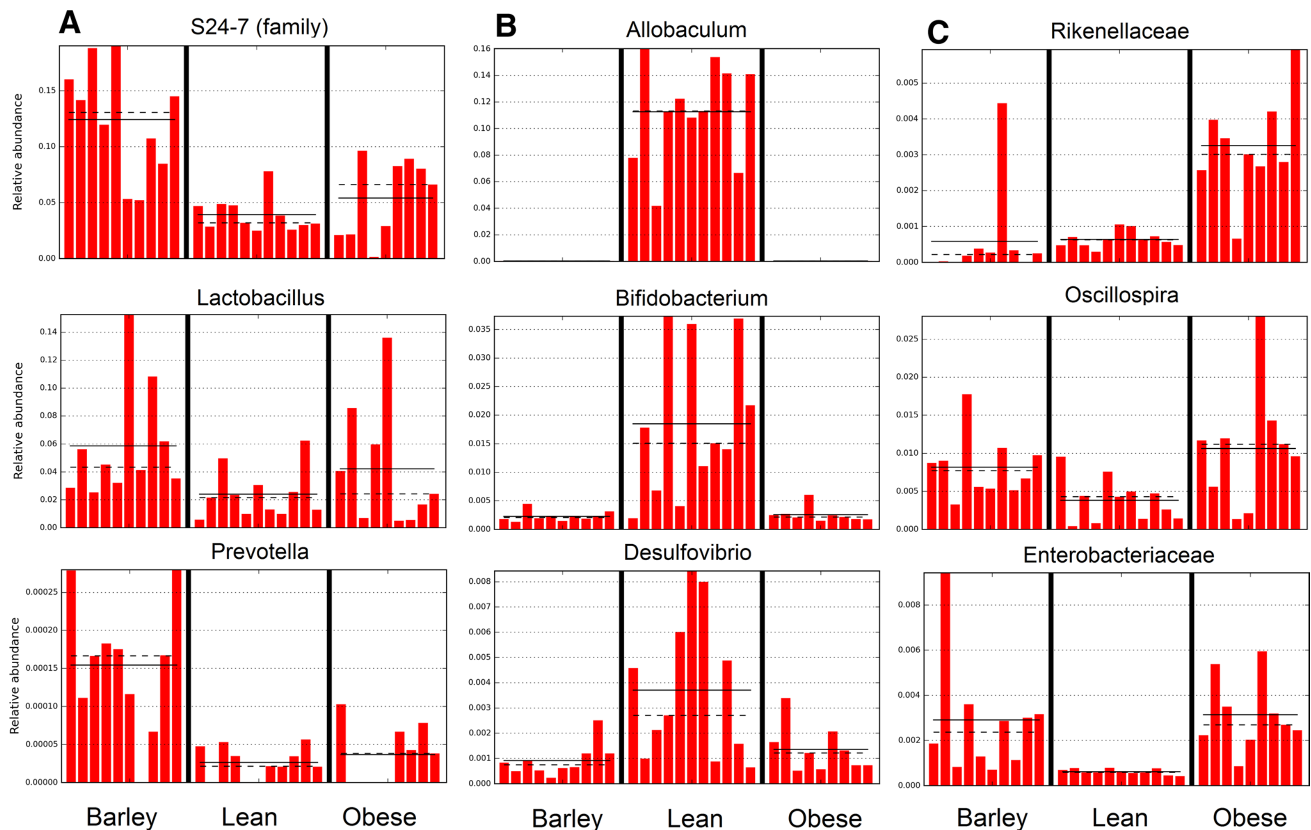

Fig. 3 Bar plots showing relative abundance of taxa that showed statistical significance according to the LEfSe method. The LEfSe method offers results accordingly to which treatment group showed the highest abundances of a particular bacterial group, and therefore the bacterial groups in A were higher in barley-supplemented mice, the bacterial groups in B were higher in lean mice, and the bacterial groups in C were higher in obese-control mice. *Dotted lines* represent medians; *straight lines* represent averages

a significant difference in species richness (i.e., number of OTUs) and the Shannon–Weaver diversity index among treatment groups, with barley group having the highest median numbers of OTUs and lean group the lowest (Table 2). Similarly, PD Whole Tree metrics were higher in the barley group compared to the lean group (Table 2).

UniFrac is a phylogenetic method that allows for determination of differences in microbial communities [37] and has been widely used in Microbial Ecology to date. In this study, PCoA plots of weighted UniFrac distances showed a distinctive cluster of all samples from lean mice independently from obese-control and barley groups (in other words all obese samples clustered together, Fig. 5a), thus suggesting that the abundance of the different bacterial groups are an important determinant to separate microbial communities based on genetic background primordially (in this case obesity). Strikingly, unweighted UniFrac distances showed a distinctive clustering of samples from each one of the three treatment groups (Fig. 5b), strongly suggesting that barley supplementation favored the emergence of microbial communities that are phylogenetically distinctive from both lean and obese. This difference between weighted and unweighted UniFrac is important to highlight because other studies from our research group have shown a clustering of all control samples (lean and obese) separated from whole-wheat-supplemented obese mice using unweighted UniFrac distances [20]. In this study, both ANOSIM and Adonis tests confirmed this clustering of samples for both weighted and unweighted UniFrac distances ($P < 0.005$), but the variation explained by this grouping was higher in the weighted analysis (52%) compared to the unweighted analysis (only 17% of the variation was explained by diet grouping). This current study and other studies [20, 21, 38] shows that both UniFrac leads to different conclusions about the effect of diet on the gut microbiota, thus highlighting the need for using both versions of UniFrac for explaining the factors associated with different microbial communities.

Predictive metabolic profile

A predictive approach of functional profiles using PICRUSt revealed that obese mice with and without barley supplementation had similar relative abundances of most gene groups compared to lean mice (over 300 gene families are

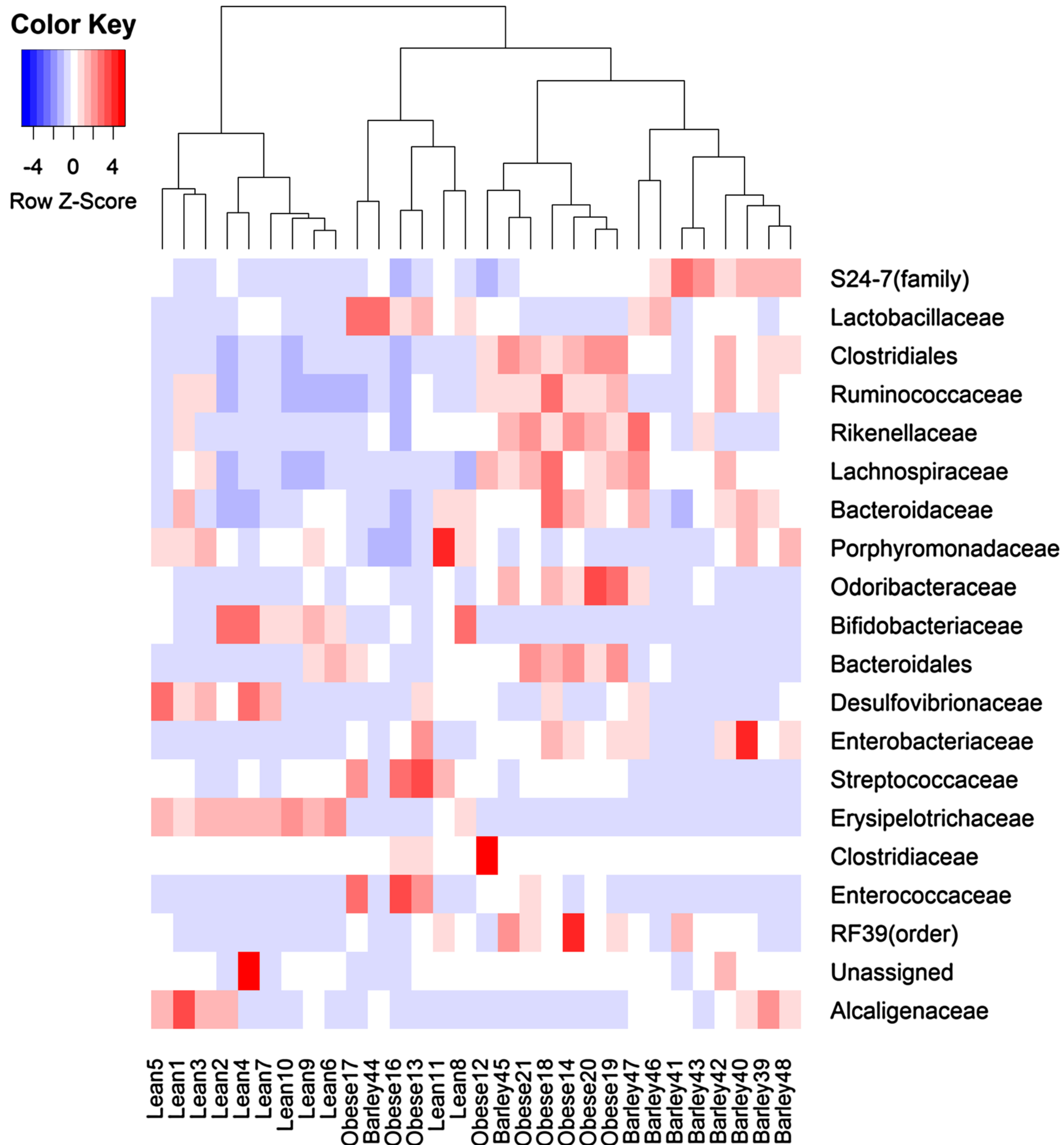

Fig. 4 Heatmap showing clustering of relative proportions of 16S rRNA gene sequences from the most abundant bacterial groups at the family level. This figure shows that barley supplementation contributes to a unique microbiota in terms of percentages of the most abundant families

Table 2 Median (minimum–maximum) alpha diversity indices

	Lean	Obese	Barley	*P* value*
OTUs	237^{a} (194–310)	304^{a,b} (154–398)	308^{b} (239–372)	0.0149
Chao1	478.8^{a} (383.6–583.9)	606.9^{a} (281.9–861.6)	528.9^{a} (359.2–782.7)	0.2948
PD whole tree	22.7^{a} (18.3–26.1)	25.9^{a,b} (20.6–30.2)	26.2^{b} (22.9–28.2)	0.0074
Shannon–Weaver	5.1^{a} (3.7–6.4)	6.4^{a,b} (3.4–7.3)	6.2^{b} (5.5–7.2)	0.0145

* *P* values come from the Kruskal–Wallis test (Mann–Whitney comparisons were adjusted with the Bonferroni method). Same superscripts indicate groups that are not statistically significantly different ($P > 0.05$)

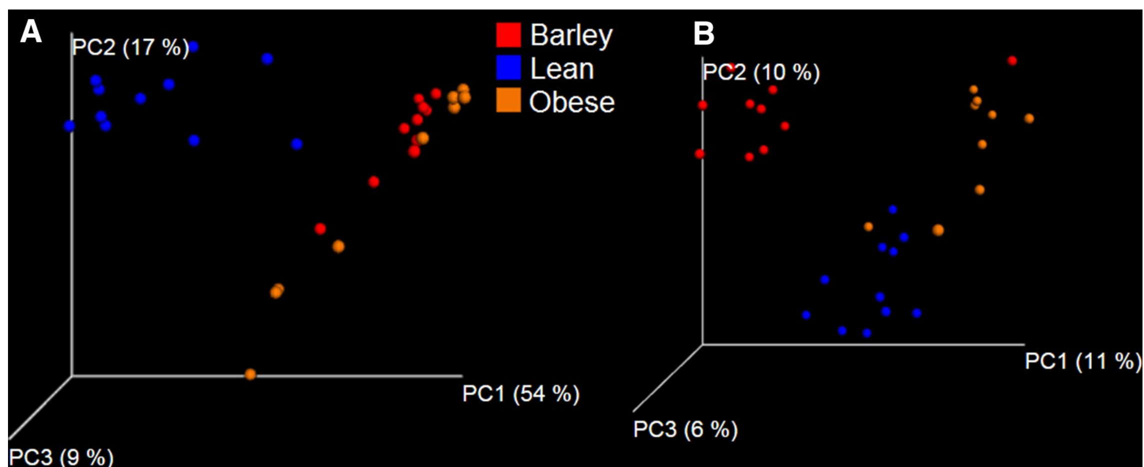

Fig. 5 Principal coordinate analysis (PCoA) plots of weighted (**a**) and unweighted (**b**) UniFrac distance metrics. Please note the discrepancy in the clustering of samples between the two versions of UniFrac (see main text for more details)

usually detected by this approach). Interesting exceptions include genes related to vitamin B6 metabolism and one carbon pool by folate (higher in barley), bacterial chemotaxis and motility proteins (higher in obese), and biosynthesis of ansamycins (lower in barley) (Fig. 6).

SCFAs in caecal contents

Butyrate and other SCFAs are important for colonic health and integrity [41] and regulation of immune system [42]. In this study, barley was not associated with lean-like butyrate concentrations in caecal contents; in fact, caecal butyrate concentrations were very similar between obese-control and barley groups and both were about 3 times higher compared to lean mice (Table 3). In contrast, the less studied succinic acid showed in this study a much lower concentration in caecal contents of barley-supplemented mice compared to all obese mice (Table 3).

Biomarkers of obesity and inflammation

Using a subset of samples, results from plasma biomarkers of obesity and inflammation showed that the levels of fasting glucose, triglycerides, total-CHL, and LDL-CHL were similar between all obese mice (Supplementary Table S2), even though barley group showed higher BMI compared to obese control (Table S1). Interestingly, barley supplementation was associated with lower levels of insulin in plasma and total cholesterol in liver compared to obese controls (Table S2).

Chronic inflammation is closely linked to obesity-related chronic diseases and dietary interventions have shown to decrease inflammation, with consequences in delaying or preventing the onset of such diseases [43]. In obesity, the overproduction of ROS promotes cell injury and production of pro-inflammatory cytokines, thus contributing to pro-inflammatory signaling pathways [44]. However, plasma levels of inflammatory cytokine IL-6 were similar between the obese groups with and without barley supplementation (Supplementary Table S3), while MCP-1 were below the range of detection (data not shown). In contrast, barley diet decreased significantly ROS levels in blood erythrocytes compared to both lean and obese controls and these results were accompanied with lower levels of protein carbonyls in plasma (Table S3). Barley diet was also associated with lower TBARS levels compared to obese controls (Table S3). This biomarker is linked to oxidative stress and adipose tissue inflammation, plays a role in obesity pathologies, and is associated with disease severity [45]. However, these results should be interpreted cautiously due to the limited plasma volumes that prevented us from analyzing TBARS and other obesity biomarkers in all blood samples collected.

The adipose tissue hormone signaling molecule leptin plays a role in energy homeostasis [46]. The absence of leptin receptor in the $Lepr^{db/db}$ mouse strain used in our study leads to obesity, hyperphagia, neuroendocrine dysfunction, and severe hyperglycemia and insulin resistance. Levels of leptin in the obese (db/db) mice were similar, as related to their genetic mutation, and higher than lean control (Supplementary Table S4). This was consistent with the similar body weight and food intake among obese animals. However, barley supplementation decreased resistin to levels similar to lean animals (Table S4). Resistin links obesity to diabetes due to its action in impairing glucose tolerance and insulin action [47].

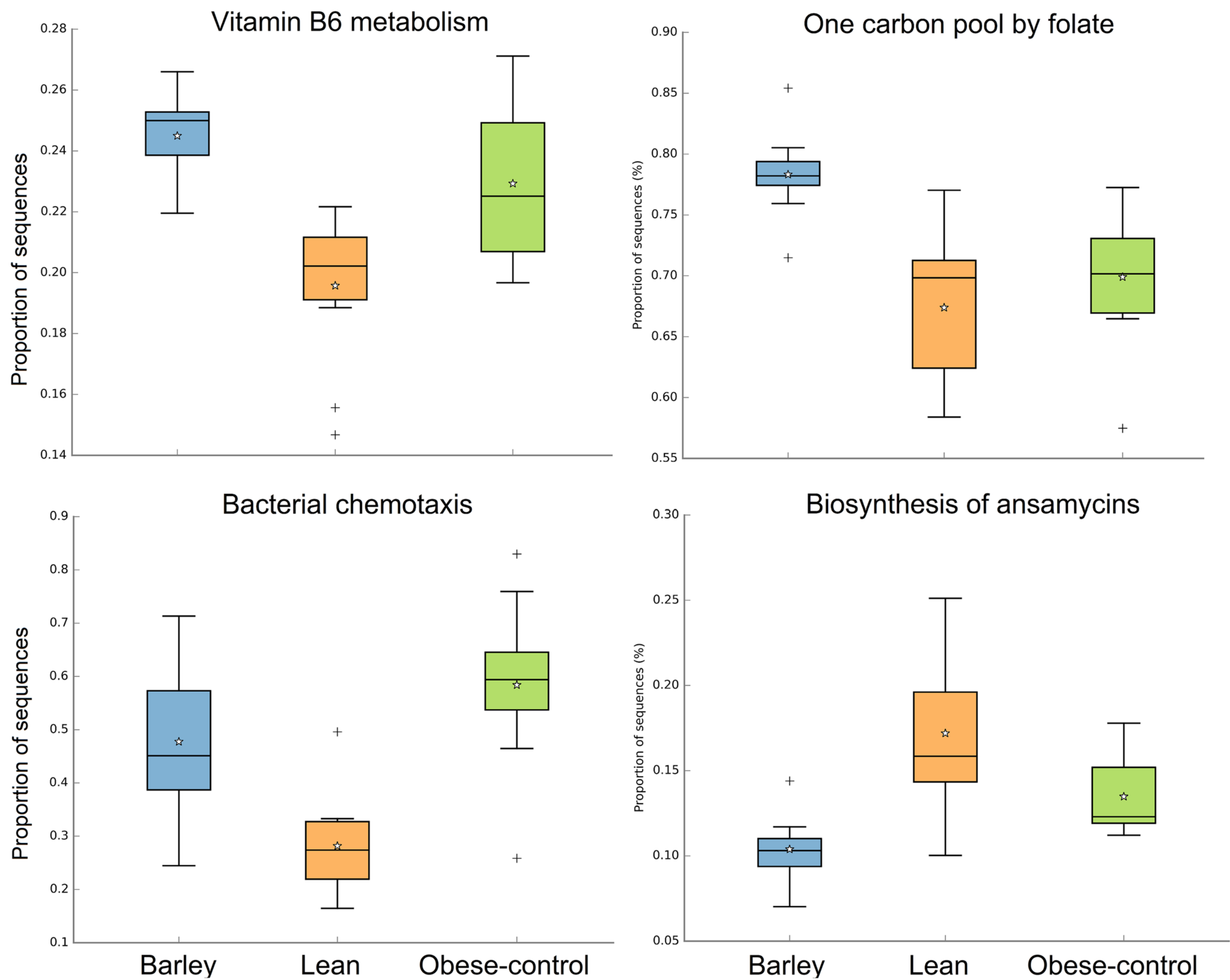

Fig. 6 Boxplots showing proportion of sequences (percentages) of PICRUSt predictive features. The comparison among groups of these four features showed statistically significant differences ($P < 0.05$, ANOVA with False Discovery Rate in STAMP). Medians are denoted by the *horizontal line* within the boxplot, averages are shown as *white stars*, and outliers are defined with the *symbol* + by STAMP

Table 3 Median (minimum–maximum) concentrations of short-chain fatty acids (SCFAs) in caecal contents (mmol/mg of caecal contents)

	Lean ($n = 10$)	Obese control ($n = 8$)	Barley ($n = 8$)	P value*
Sodium butyrate	0.9^{a} (0.2–2.7)	2.9^{b} (1.5–4.3)	2.8^{b} (2.1–4.7)	0.001
Succinic acid	39.8^{a} (15.3–97.6)	22.9^{a} (3.9–71.2)	3.9^{b} (0–7.8)	<0.001
Acetic acid	10.2^{a} (7.7–26.3)	12.01^{a} (8.3–18.7)	10.4^{a} (8.5–14.9)	0.769
Oxalic acid	15.1^{a} (6.7–18.9)	14.6^{a,b} (8.8–28)	9.7^{b} (7.8–13.2)	0.038

* P values come from the Kruskal–Wallis test (Mann–Whitney multiple comparisons were adjusted with the Bonferroni method) in PAST. Same superscripts indicate lack of statistical significant difference ($P > 0.05$)

Discussion

Barley is a nutritious, heart-healthy whole grain that can reduce blood glucose levels and the risk of diabetes [48], while at the same time providing nutrients such as fiber, protein, vitamins, and minerals [12]. The beneficial effects of barley and other cereals are due to different (often intertwined) mechanisms including a delayed intestinal absorption of glucose and lipids, inhibition of absorption–reabsorption of cholesterol and bile acids, and increased excretion

of bile acids [13, 14, 49]. It is also possible that the high viscosity of β-glucan (very high in some varieties of barley) solutions increases the viscosity of the intestinal contents thus reducing absorption of cholesterol [50, 51], and that β-glucan is preferentially fermented in the large intestine by specific microorganisms that produce SCFAs thus impeding cholesterol biosynthesis [13].

This study shows that body weight was not affected by consumption of barley in db/db mice. Interestingly, the BMI in barley group was higher compared to obese control and lean control but this phenomenon was not due to body fat accumulation since all obese mice (with and without barley) had similar adiposity indexes. This is important considering the role of body fat in obesity-related chronic inflammation and the development of insulin resistance [52]. Consumption of barley was also associated with higher cecum weight and cecum content compared to obese-control group, which may be related to the content of dietary fiber in barley (hulled barley contains 17.3% of dietary fiber according to the National Nutrient Database, USDA 2016) [53]. The biological effects of dietary fiber include inducement of caecal fermentation and increase in cecum and stool weights [54]. The increase in cecum contents has been related to β-glucans in barley. Barley diets with 3% β-glucan increased significantly caecal digesta mass and individual and total short-chain fatty acids compared to 0% β-glucan barley [55]. In addition, β-glucans have been shown to cause changes in caecal microbiota and profile of SCFA in rats fed high-fat diets [56].

The metabolism of barley and other food ingredients inside the digestive tract is closely associated with the gut microbiota. Interestingly, several publications have demonstrated an effect of barley on the fecal microbiota of healthy [15] and mildly hypercholesterolemic human subjects [57]. Barley has also been shown to modify the gut microbiota of horses [16], growing pigs [58], and rats fed high-fat diets [56]. To our knowledge, however, there are no studies that have investigated the effect of barley on the gut microbiota of genetically obese mice.

LEfSe analysis revealed interesting differences in bacterial membership in barley-supplemented mice. For example, the poorly studied group of bacteria S24-7 (Candidatus Homeothermaceae) within the Bacteroidetes was higher in barley-supplemented mice. This group is particularly interesting because a recent study also showed that whole wheat was associated with more S24-7 [20], suggesting that this group is susceptible to exposure to whole-grain cereals (and probably other nutrients too) irrespective of the source. In support of this hypothesis, Serino et al. [59] and Shen et al. [60] showed that the abundance of S24-7 is susceptible to diets enriched in gluco-oligosaccharides and dietary protein, respectively. Interestingly, a recent genomic analysis of S24-7 revealed that this group is actually composed by three trophic guilds each roughly defined by increased amounts of enzymes involved in the degradation of specific carbohydrates [61]. Another important group that showed increased abundance in barley-supplemented mice was *Prevotella*, a relatively well-studied group capable of fermenting β-glucans [62] and other carbohydrates [63]. In fact, one seminal study showed that *Prevotella* was the bacterial group most closely associated with long-term diets consisting of carbohydrates [11]. It is important to recall that S24-7 and *Prevotella* are only two members of Bacteroidetes, a vast heterogeneous group of microorganisms [64] that, as a whole, have been associated with body weight conditions. For example, one study showed that Bacteroidetes was lower in obese compared to lean mice [65] although conflicting reports where obese individuals have more Bacteroidetes have been published [66, 67]. These conflicting results are due in part to the heterogeneity within the phylum [64]. Another group of interest that showed higher abundances in barley-supplemented mice is the family Mogibacteriaceae, a member of the Clostridiales. Interestingly, this group has been associated with a phenotype characterized by a high frequency of bowel movements and also a lean body type [68]. Another study showed increases in this group in feces of mice upon cold exposure [69].

Different members of the gut microbiota have historically been associated with beneficial properties (e.g., *Lactobacillus* and *Bifidobacterium* spp.). In this study, lean mice had higher fecal *Bifidobacterium* compared to obese mice with and without barley supplementation, while *Lactobacillus* was lower in lean mice and higher with barley. The genus *Allobaculum* was also found to be higher in lean compared to both obese groups. *Allobaculum* is a poorly studied group of microorganisms that correlate negatively with adiposity [70]. The finding of similar bacterial populations (e.g., *Bifidobacterium* and *Allobaculum*) among all obese mice (irrespective of diet) may suggest resilience upon dietary modification and that the population control of these groups is mostly related to host genetics. However, currently we know little about the heritability of gut microbes [71] and therefore these are only speculations that need further evaluation. Diversity analyses revealed that all obese mice (with and without barley intake) showed similar values with regard to alpha diversity measurements (numbers and types of bacteria) and that these values were relatively higher compared to lean controls. This is interesting because a more diverse ecosystem is usually associated with more stability and health in adult animal hosts including humans [72].

In this study, caecal butyrate concentrations were similar between obese-control and barley groups and both were about three times higher compared to lean mice. In high contrast, two recent studies from our research group showed higher and lower concentrations of caecal butyrate during supplementation of whole wheat and quinoa, respectively, compared to obese controls [20, 21]. The fact that different

food ingredients have distinct effects on the gut microbiota has also been shown in pigs fed wheat and barley [73]. While our current study did not aim to identify the specific microbial groups responsible for production of butyrate in the gut, it does offer valuable information for future studies of health benefits. In particular, butyrate is important because it has been shown to improve insulin sensitivity and energy expenditure in mice [74] and its close connection with the microbiota–gut–brain axis [75].

Other SCFAs aside butyrate are also of interest for the biomedical community. For example, in this study the less studied succinic acid showed a much lower concentration in caecal contents of barley-supplemented mice compared to obese-control mice. Similarly, this compound was also lower in caecal contents of whole-wheat- [20] and quinoa-supplemented obese mice [21]. Succinic acid can have deleterious effects such as inhibition of large intestine motility and depression of the proliferation rate of colonic epithelial cells [76]. Interestingly, Jakobsdottir et al. [77] showed an increase of this compound in rats fed a high-fat diet and a positive effect of dietary fiber at lowering its concentrations. Based on these observations, the fact that barley helped lower the concentration of this compound in this current study (compared to obese controls) could be considered a positive effect; however, the question remains as to why metabolically healthy lean mice had similar (in fact higher although not statistically) concentrations of succinic acid compared to obese (Zhong et al. [56] showed that the concentrations of succinic acid were correlated with the abundance of *Clostridium* and *Akkermansia*, thus suggesting that the concentrations of succinic acid may depend on a consortium of different organisms, a well-known phenomenon for other SCFAs such as butyrate [78]). This is important because high concentrations of succinic acid have been shown in other microbial ecosystems such as the human vagina in the presence of harmful bacterial vaginosis [79]. Interestingly, different studies suggest that the abundance of succinate increases during disruption of normal gut microbial homeostasis, thereby promoting infection by bacterial pathogens [80]. Our results show that barley consumption is associated with much lower concentrations of caecal succinic acid and this deserves scrutiny in further studies.

Barley has one of the lowest glycemic indexes thus offering a good dietary alternative for patients with diabetes, obesity, and associated disorders. In this study, consumption of barley supplementation was associated with lower levels of insulin and total-CHL in liver compared to the obese-control mice. This result may be due to the high viscosity of β-glucan solutions which increases the viscosity of the intestinal contents thus reducing absorption of cholesterol [50]. These results are consistent with a study in which a diet containing ~6% β-glucan from barley flour decreased liver lipids in Zucker diabetic fatty rats [81]. In contrast, another study reported that barley diet (independent of the β-glucan contents) failed to improve the levels of liver lipids in rats fed high-fat diet [82]. This suggests an important difference between genetically obese and diet-induced obese rodent models as shown elsewhere [83].

In this study, barley supplementation decreased plasma resistin, insulin, and protein carbonyls compared to obese controls. This is consistent with reports linking resistin to obesity, inflammation, diabetes, and cardiometabolic diseases due to the effect of resistin in impairing glucose tolerance and insulin action [44, 84]. However, even though resistin plays an important regulatory role in the inflammatory response [84], our results showed that IL-6 was similar in all obese mice. This might suggest that, by the end of the study, mice were not old enough for diabetes progression to raise the inflammatory cytokines as it has been shown in older db/db mice [85, 86]. The effect of barley supplementation on circulating resistin levels might be in part related to its content of folic acid (19–23 μg/100 g) (USDA nutrient database [53]). One study reported that plasma resistin was reduced in obese/diabetic mice by 25% after 4 weeks of supplementing with 71 μg folic acid/kg [87]. In addition, resistin and resistin-like molecules are also produced in the intestinal tract and secreted into circulation [88]. This leads to a relatively unexplored field of research on gut microbiota–intestinal tract–resistin secretion axis. Likewise, the lower levels of oxidative stress in barley-supplemented group quantified through ROS in erythrocytes might indicate changes in gut pathogenic bacteria [89]. The production of ROS by the mitochondria has been recognized as an important target of pathogenic bacteria. An imbalance in microbiota can regulate the mitochondria interaction with the host and may result in a pathogenic state in which excessive mitochondria ROS production regulate gut epithelial barrier acting as feedback loop that aggravates the microbiota–host interaction [89]. Therefore, dietary strategies that decrease ROS might be the cause or consequence of an improved microbiota balance.

Limitations

Barley is an interesting option as a functional food for obese and diabetic patients [13, 14] but there are differences among varieties of barley and processing regimes, thus affecting nutrient composition and consequently any potential health effects on the consumer [90]. Second, each meal and dietary ingredient (e.g., wheat, barley) possess their own unique complex microbiota [91] that when consumed could exert changes on the native gut microbiota of the host. In this study, the barley-associated microbiota was not assessed (i.e., diets were offered without irradiation in an effort to simulate a scenario

that reflects barley consumption in real life). Third, we acknowledge that diet supplementation with approximately 88% barley is not relevant to human nutrition. Our study can nonetheless lead to another study in which different doses of barley can be compared to find out the minimum doses in which barley intake is able to promote changes in the gut microbiota. Finally, in this study we used a genetic model of obesity, which often diverges from systems of diet-induced obesity as shown elsewhere [83]. In this regard, diet-induced models of obesity are useful mainly because diet plays an important role in human obesity. However, diet-induced obesity has the disadvantage of altering feeding patterns [92]. Also, different protocols have been published regarding the length of feeding time and the type of diet (i.e., constituent ingredients) to make the animals obese, without clear guidelines [93–95]. Moreover, there are cases of resistance to diet-induced obesity, a poorly studied phenomenon [96]. Finally, there are already studies that have investigated the effect of barley on diet-induced obesity animal models [56].

Conclusions

In this study, barley intake was associated with higher abundances of *Prevotella*, *Lactobacillus*, and S24-7 as well as divergent proportions of genes related to vitamin B6 metabolism and biosynthesis of ansamycins. Barley supplementation did not have an effect on caecal butyrate compared to obese controls but succinic acid was much lower compared to lean and obese controls. The potential health effects and physiological benefits of barley incorporation in diet are supported by a reduced concentration of plasma insulin, resistin, and decreased oxidative stress measured through levels of ROS in erythrocytes compared to obese controls receiving a standard diet. Thus, the relationship between these important biomarkers to changes in gut microbiota and microbiota metabolites modulated by barley are worth investigating in future studies to aid prevent or treat obesity-related disorders.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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