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Gut Microbiome and Its Role in Obesity and Aging in C57BL/6J mice

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ABSTRACT

The gut microbiome has been found to be associated with obesity, type 2 diabetes and many other diseases. Many studies have shown microbial composition changes with obese status or switching of diets. However, few of them have investigated the long-term microbial changes in subjects under the same environmental factors. This study examined gut microbiome changes in multiple aspects with well-controlled diet-induced obese mice models and demonstrated the following: there were great variations in gut microbiome composition and diversity in the same strain of inbred mice under the same environment and diet; certain lineages of bacteria were associated with digestive efficiency; gut microbiome changes were dose dependent on different levels of calorie restriction; gut microbiome were relatively stable in adult aging under fixed feeding regimen; weight cycling through manipulating the amounts of diet intake could have differential effects on microbiome composition and specific categories of bacteria; and microbial compositions at a younger age were different between short-lived and long-lived mice. In summary, this study provides substantial insight into the roles of gut microbiome in obesity, calorie restriction and aging with well-controlled experimental subjects and conditions. These results also provide a rationale for future interventional study and subsequent clinical application in the prevention and treatment of obesity, as well as potential strategies for promoting longevity.

Keywords: gut microbiome, obesity, aging, C57BL/6J, mice

DEDICATION

To my grandmother, Maihua Wang, for the selfless and unconditional love. To all of my family for believing in me and supporting me.

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LIST of ABBREVIATIONS

BW	body weight
CR	calorie restriction
DE	digestive efficiency
DIO	diet-induced obesity
EO	ever obese
FI	food intake
SCFA	short chain fatty acids
OTU	operational taxonomic unit
OWL	obese weight loser
OWLM	obese weight loser moderate
WC	weight cycling/weight cycler

INTRODUCTION

The prevalence of obesity has been increasing over the last few decades and is a major burden to public health and healthcare resources. Obesity is related to the imbalance between energy intake and energy expenditure and is influenced by host genetic background and environmental or lifestyle factors such as diet. Recent insight suggests that the intestinal microbiome should be considered as a subset of genetic factors, together with host genotype and lifestyle (energy intake and expenditure), contributing to variations in adiposity [1].

Studies have found marked interpersonal differences in species level diversity of the gut microbiota [2-5], which might be explained by factors including diet [6], the use of antibiotics [7], the genetic background of the host [8] and others. However, one study of 59 mammalian species revealed that their fecal microbiota clustered according to diet rather than host phylogeny [5]. Later studies found that high-fat diet, not the obese state, accounts for the altered microbial communities in mice [6], and that the structure and function of the gut microbiome were significantly associated with high-fat diet feeding [9]. But it is poorly understood how exactly diet interacts with gut microbiome in their association with obesity.

Calorie restriction, without malnutrition, has long been shown to improve health and increase lifespan in multiple species [10], but the underlying mechanism is still not well understood. Reduced cellular divisions, lower metabolic rates, reduced production of free radicals and hormesis have all been suggested as possible explanations

[11-15]. Recent studies have found that gut microbiome might be altered during CR. It was reported [16] that short-term CR-induced weight loss in human adolescents had an impact on the composition of the gut microbiota: subjects with greater weight loss had significantly different changes in gut microbiota composition compared to those with less weight loss. Nonetheless, there is little information on how the gut microbial communities respond to sustained CR.

Although extensive studies have been conducted to study microbiota in adults, investigation into structural changes and compositional evolution from young to the elderly is rarely done. The composition of the intestinal microbiota in older people (>65 years) is extremely variable among individuals [17] and differs from the core microbiota and diversity levels of younger adults [17, 18]. It was hypothesized that the aging process could affect the gut microbiota after age 65 (which is the common criteria for defining elderly), and that the aged microbiota stabilizes at the age of 75–80 years [18]. However, evidence is scarce regarding microbial changes with the natural aging process.

The overall objective of this proposed research is to understand gut microbiome changes in diet-induced obesity, subsequent weight loss through calorie restriction and weight cycling and aging.

LITERATURE REVIEW

The human intestine contains ~10 to 100 trillion microbial cells, which is 10 times more cells than the human body. Microbes in the human gut undergo selective pressure from the host and environmental factors (such as nutrition), as well as from microbial competitors, which leads to a homeostasis of the ecosystem, with some in high abundance and others in low abundance [19]. The gut microbiota has important metabolic

functions, such as detoxification, micronutrient synthesis, fermentation of indigestible food substances and assistance in the absorption of certain electrolytes and trace minerals [20-22]. It has been suggested that gut microbes help to break down otherwise indigestible foods and contribute to energy harvest and obesity [1, 23]; for example, microbial fermentation of polysaccharides to short chain fatty acids (SCFA) can account for up to 10% of human daily caloric intake [24]. The energy from microbial fermentation will positively contribute to adiposity given that even small, sustained changes in energy balance over a long time can result in significant changes in body weight (BW) [25]. It was shown that *ob*/*ob* mice are more effective at harvesting or acquiring calories from food during digestion than their lean siblings, and this feature is transmissible (through bacteria transplant) to germ-free recipients, resulting in greater adiposity [1]. Furthermore, a bacterially related factor, as well as the abundance of certain bacteria species, has been suggested to be responsible for high-fat diet–induced obesity [26], since germ-free mice fed a high-fat diet gained the same weight as those with low-fat diet [11]. Studies have consistently found that the proportional changes of *Bacteroidetes* and *Firmicutes*, which are the two dominant bacterial phyla in humans and many animals, are associated with obesity [27, 28]. Similar differences in this bacterial ratio have been observed in human studies between obese and lean subjects [2, 3, 29], but no correlation between BMI and the *Firmicutes*/*Bacteroidetes* ratio was observed in humans [30]. On the contrary, despite weight loss, there were no changes in the relative counts of the *Bacteroides* spp. or the percentage of *Firmicutes* [29, 31]; *Bacteroidetes* was significantly correlated to weight loss but not to total caloric intake [2], thus

suggesting that it is not necessarily just the ratio of *Firmicutes* and *Bacteroidetes* that is important but rather the amount of SCFA produced.

For a given individual with gut microbiota altered by obesity status or by high-fat diets, the aberrant microbiota can affect different physiological mechanisms regulating body energy metabolism, lipid homeostasis and immune function. But currently, there is no consensus as to whether the gut microbiome plays a causative role in obesity or is secondary in response to the diet associated with obesity. In addition to the commonly agreed on abundance of *Firmicutes* and *Bacteroidetes*, *Actinobacteria* was also found to be abundant in obese animals [32]. *Lactobacillus* species, which are widely used as growth promoters in the farm industry, were recently found to be increased in some obese individuals compared to lean individuals [33]. Moreover, high-fat diet–fed mice treated with antibiotics were found to be partially protective against diabetes, which was proposed to be caused by the alteration of gut microbiota composition [7]. On the other hand, prebiotics have been shown [34] to increase *Bifidobacteria*, lower endotoxaemia, improve glucose tolerance and regulate body weight gain in high-fat diet–fed mice. One study suggested that supplementation of both *Bifidobacterium* and conjugated linoleic acid improved fatty acid composition of the host liver and white adipose tissue significantly over either supplemented alone, indicating that dietary manipulation represents a realistic target for modification of the fatty acid composition and proinflammatory cytokine profile of the host tissues [35]. While obesity and gut microbiome changes are possibly secondary to high-fat diet feeding, it is unknown whether there is any causality between changed gut microbiota and obesity. In our previous work, we observed large variations in their responses to prolonged high-fat diet

feeding, with over twofold differences in their body weight and over fivefold differences in fat mass; however, these mice were an inbred C57BL/6J strain, of the same age, receiving the same diet and singly housed. We found the average daily energy intake was significantly correlated with body weight gain. However, in the mice receiving the same amount of food, large variations in body weight still existed.

While calorie restriction has been an effective way of losing weight and beneficial in metabolic, hormonal and functional changes for obese individuals, calorie restriction without malnutrition has also been shown to prolong lifespan in mammalian and invertebrate species [36, 37]. Reduced metabolic rate or oxidative metabolism is one of the possible explanations for the anti-aging effects of CR [38]. Additionally, CR is hypothesized to lessen oxidative damage by reducing energy flux and metabolism [39]. Reduced energy intake by CR results in loss of body mass and a reduction in metabolism. The human body was described to have a "metabolic adaptation," exemplified by a reduction in the metabolic rate concomitant with the decreased body mass [40]. This adaption may be caused by many factors, including genetic, metabolic, social or behavioral [38]. Nonetheless, it is speculated that it is the reduction in food intake (FI), and not a reduction in fat mass, that is the beneficial component of CR [41]. Another study suggested [42] that the changes in gut bacterial community structure during diet-induced weight loss are a reflection of the effects of reduced nutrient load rather than the actual weight loss. A possible explanation is that the gut or residing microorganism senses alterations in nutrient availability and subsequently modulated the nutrient absorption. The presence of gut microbiota is also found to enhance the supply of a nutrient source (ketone bodies) during fasting in germ-free mice [43]. Furthermore,

decreased plasma concentrations of inflammatory cytokines were reported in mice under CR [44], while gut microbiota is significantly associated with plasma concentration of lipopolysaccharide (LPS) [45]. One study [46] reported several gut microbiota family changes after gastric bypass surgery, which mimics CR by restricting the amount and types of food ingested, albeit following surgery medication exposures and potentially altered nutrient delivery for the lower intestine. Gut microbiota composition changes rapidly after feeding a high-fat diet [32]; however, it was suggested that the capability of increasing energy harvesting associated with the microbiota profile is not constant after prolonged exposure, because the fecal energy decreased in obese animals over a short period after switching to high-fat diet, while lean animals remained stable [47]. It is unclear whether an association exists between the gut microbiome composition changes and sustained CR. Additional information concerning these aspects is needed.

There have been few studies looking at the gut microbiome changes in individuals with aging. Yatsunenko *et al.* [48] found age-associated changes in the genes of the gut microbiota involved in vitamin biosynthesis and metabolism and that bacterial complexity increased with age in individuals of different geographic locations. Claesson *et al.* [49] reported intestinal microbiota differences in four residence locations (community-dwelling, outpatient, short-term rehabilitation hospital care and long-term residential care). They observed correlations between gut microbiota and food diversity categories, as well as several health status measurements [49]. Additionally, the core microbiota of elderly subjects was distinct from younger adults, with a greater proportion of *Bacteroidetes* and *Clostridium* and a lesser proportion of bifidobacteria [50, 51]. One study [18] observed that the microbial composition and diversity of the gut ecosystem of

young adults and 70-year-old people is highly similar yet differs significantly from that of centenarians, with centenarians having a more than tenfold increase in *Eubacterium.limosum*, the signature bacteria of long life that has an anti-inflammatory property [52]. However, these observations were cross-sectional instead of longitudinal and might be confounded by many other factors. It was evidenced that the ageing process is deeply associated with the structure of the human gut microbiota and its homeostasis with the host's immune system [18]. *Proteobacteria* were seen as "pathobionts" in late stage of life [53, 54], which might overtake mutualistic symbionts and induce pathology. The abundance of some bacteria, like *Clostridiales* [30], has been found to be negatively related to age. However, it is unknown whether the gut microbiota can influence the ageing process contrarily.

For obese individuals, losing weight is possible, but the maintenance of weight loss for a prolonged period is seldom successful [55]. As a result, repeated weight losses and regains (yo-yo dieting) have become a common pattern for obese individuals [55-57]. Although the bulk of observational epidemiologic research shows an association of weight variability with morbidity and mortality, these observations might be confounded by a number of issues – for instance, unintentional weight loss and concurrent diseases [58]. A very recent study with a relatively small sample size concluded that weight-cycled mice switching between high-fat and low-fat diets had no significant difference in lifespan as compared to low-fat diet–fed controls, while being overweight and eating a high-fat diet led to a significantly shorter lifespan [59]. On the other hand, weight cycling may have some "temporarily" healthy benefits in decreasing resting metabolic rate [60] and serum glucose and insulin levels [61]. The major objective of a longevity study, from

which the animals proposed to be used in this proposal derive, is to determine whether repeated bouts of weight loss and regain among obese rodents increase or decrease mortality rate relative to maintaining an obese weight or to achieving sustained weight loss. While the gut microbiome is associated with diet intake and possibly the obese state, we hypothesize that gut microbiome should be correlated with food intake changes; however, there are no extant data in this regard.

The Human Microbiome Project (HMP) has been launched worldwide [62] to identify new ways of determining health and predisposition to diseases by gut microbiome and optimizing its performance in the context of an individual's physiology [62]. There are several important questions to be answered by the HMP project, some of which would not be feasible in the short term – for example, the stability and resilience of an individual's microbiota through lifespan and whether there is an identifiable "core" microbiome shared by the population. Literature demonstrates [32, 63] that defining the effects of diet and age on gut microbiota composition and function will be essential for analyzing and interpreting the massive data sets generated in the different meta-genomics projects worldwide.

The animal model to be used in this study is diet-induced obese mice, which more closely reflect the situation in humans than genetically mutated animals (though these animals are still inbred). The high-fat content of the diet used takes up 45% of the energy content, which is close to typical calorie intake from fat in the U.S. [64]. Furthermore, there is considerable similarity between human and mouse distal gut microbiotas at the division level [27]. Many factors in unrestricted human subjects, such as lifestyle-related factors like exercise level and energy intake, genetic background, habitation and overall

fitness, which are more important in causing and maintaining obesity, will be controlled in this study. To our knowledge, there have been no reported long-term longitudinal evaluations of the gut microbe composition, and the relatively shorter lifespan of mice would enable us to track the aging effects. Overall, this unique inbred, non-mutant, singly housed mouse model with controlled diet and long-term sample collection would provide insightful and comprehensive data in addressing the questions that arise from the specific aims.

EXPERIMENTAL AIMS AND HYPOTHESES

The mice longevity project has provided invaluable data and opportunities to explore many aspects of science related to diet-induced obesity, calorie restriction and aging. By utilizing the fecal samples and physiological measurements, we will be able to test many hypotheses relating to gut microbiome compositions during diet-induced obesity, calorie restriction and aging. The overall objective of this dissertation is to investigate gut microbiome compositions and changes in diet-induced obesity variability, digestive efficiencies, different levels of calorie restriction, repeated weight loss and regain cycles and the aging process, as well as longevity. Specifically, there are three experimental aims and seven null hypotheses, as follows:

Experimental Aim 1

This experimental aim will assess fecal microbial diversity in genetically similar mice under the same feeding regimen but differing dramatically in body weight or food intake. There are two hypotheses under this experimental aim:

Hypothesis 1a. There will be no differences between fecal bacteria populations in hosts with significantly different levels of adiposity under the same feeding regimen.

Hypothesis 1b. Fecal bacteria compositions (or specific strains of bacteria) are not associated with digestive efficiency (DE) or fecal energy density.

Experimental Aim 2

Experimental aim 2 will test fecal microbiota changes after changes in the amount of food intake in both the short term and the long term. There are three hypotheses under this experimental aim:

Hypothesis 2a. Fecal bacteria compositions (or specific strains of bacteria) would have similar changes in mice under different levels of caloric restriction compared with *ad libitum*-fed mice.

Hypothesis 2b. Fecal bacteria will not respond to chronic diet changes.

Hypothesis 2c. Fecal bacteria composition will be the same after the mice go through repeated weight loss and regain cycles through calorie restriction and *ad libitum* refeeding.

Experimental Aim 3

Experimental aim 3 will evaluate longitudinal fecal microbial stabilities with aging under a fixed feeding regimen. There are two hypotheses under this aim:

Hypothesis 3a. Fecal bacteria composition will be stable under a fixed diet regimen independent of time effect.

Hypothesis 3b. Baseline fecal composition (or specific strains of bacteria) will be the same at baseline between short-lived and long-lived mice.

METHODS

The study animals are from an ongoing NIH-funded research project (R01AG033682, PI – Allison DB, body composition, energetics and longevity). This proposal is designed independently and is not related to the original R01 research project. This proposal will collect non-invasive samples only (fecal pellets) and will use part of the body weight, food intake and body composition data from the R01 research project.

Study Animals

375 C57BL/6J male mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age and singly housed with high-fat diet feeding (45% kcal fat and 20% protein; D11112301, Research Diets, New Brunswick, NJ) at 8 weeks of age and thereafter. The heaviest 2/3 mice (n=252) were randomized at 11 months of age into four groups (with the same high-fat diet): Ever Obese (EO, n=43) – continued ad libitum (AL) feeding; Obese Weight Losers (OWL, n=42, around 40% restriction of the EO) – diet restricted to a body weight similar to low-fat–fed animals (low-fat diet, 10% kcal fat); Weight Cyclers (WC, n=82) – diet restricted, followed by AL refeeding cycles over the course of life with average body weight between the EO and OWL groups; and Obese Weight Losers Moderate (OWLM, n=83, around 20% restriction of the EO) – diet restricted to approximate a stable, average body weight in the middle of EO and OWL. Additionally, 10 of the culled 1/3 ever lean (CULL) mice with lowest body weight are maintained on the AL high-fat diet feeding for comparison with other groups. For detailed projected body weight curves, please refer to **Figure 1** below.

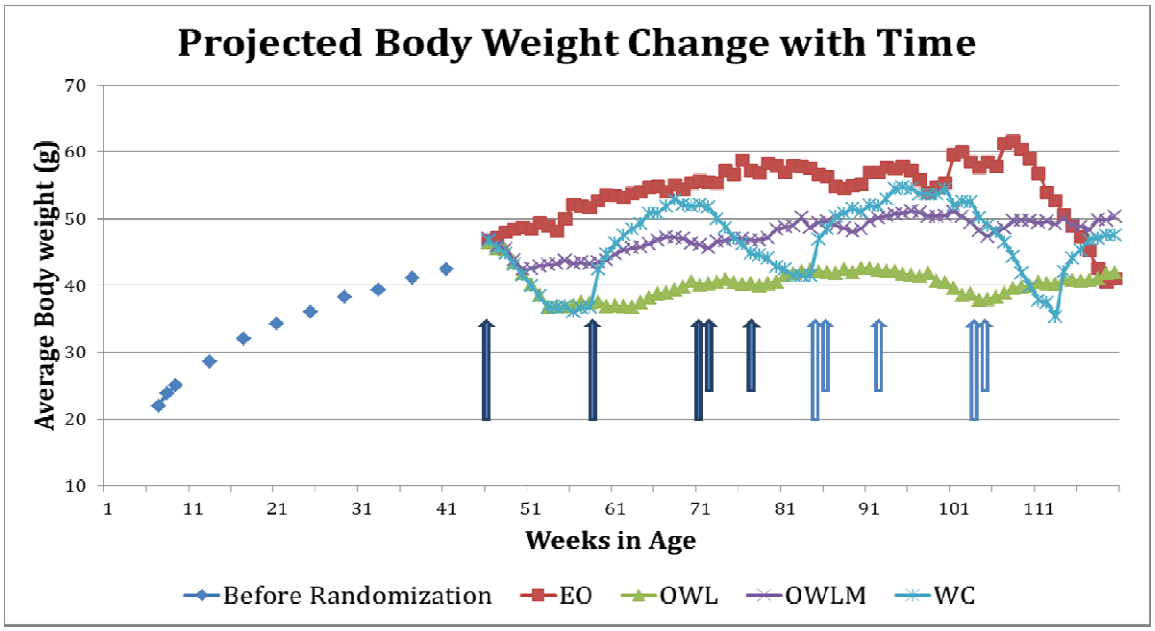


Figure 1. Time point of sample collection in the study design. (Note: the data used in this chart are from a previous study cohort of the same design; solid blue arrows represent sample collection for microbiome sequencing; hollow blue arrows represent additional sample collection for fecal energy output measures; shorter arrows represent sample collection for WC only; low-fat–fed animal and CULL group are not shown.)

C57BL/6J is the most widely used inbred mouse strain and is susceptible to diet-induced obesity and other metabolic diseases [65]. The definition of an inbred strain is that mice are produced using at least 20 consecutive generations of sister x brother or parent x offspring matings, or mice are traceable to single ancestral pair in the 20th or subsequent generation. They are as genetically alike as possible and homozygous at virtually all of their loci [66]. Male C57BL/6J mice have a mean lifespan of 25~27 months [67]. The 45% high-fat diet was to mimic the typical western diet. All work and procedures are approved by the UAB Institutional Animal Care and Use Committee.

Sample and Data Collection

Body weight and food intake are recorded weekly from the beginning of this study. Non-invasive body composition measurement (fat mass and fat-free mass, by

quantitative magnetic resonance, QMR, Echo MRI^{TM} 3-in-1 V2.1; Echo Medical Systems, Houston, TX) is conducted at each weight rise and fall of the WC for all mice. Mortality events were recorded to the nearest day, with information regarding the natural/spontaneous death versus euthanized animals for moribund conditions recorded. Gross necropsy is conducted upon death of the mice.

Fresh feces (2~4 pellets) were collected before randomization (11 months of age) and with each weight change cycle (based on the rise and fall of WC mice, ~ 2 month intervals, Figure 1) for all groups and frozen at -80ºC until microbiome analysis. Fecal pellets contain bacterial populations resembling those present in the lower gastrointestinal tract and may therefore provide a convenient sample source [4]. Samples will be collected until 50% mortality of all mice, which roughly covers a period of at least one year (age from 11 to 23 months). Proposed sample collection and utilization were shown in **Table 1**.

Prior to 44 weeks of age, all of the mice received exactly the same treatment, including *ad libitum* feeding of a high-fat diet. Due to the fact that this proposal was conceived later than the beginning of the original R01 project, there were no samples collected from an early age. However, the samples collected at 44 weeks could be used as a baseline to investigate the relationship between gut microbial diversity and individual adiposity variations, effects of calorie restriction on gut microbiome and gut microbiome stabilities with aging. To investigate the longitudinal changes of gut microbiome composition changes, only samples from those mice that survived longer than 104 weeks were selected. Thus 20 or 10 samples, which were alive at 104 weeks of age and did not

have any severe health consequences, such as ulcerative dermatitis, were randomly selected for microbiome sequencing.

Table 1. Proposed time points and sample sizes for collection.

Group	Time points and mice ages in weeks									
	0	1	2	3	4	5	6	7	8	9
	44w	61w	75w	76w	79w	88w	89w	91w	103w	104w
EO	20	10	10			10			8	
OWL	20	20	20			20			20	
OWLM	30	20	20		70	20			20	
WC	30	20	20	20	20	20	20	20	20	20
EL	10	5	5			5			5	

Note: Underlined numbers represent samples collected for fecal energy output measures in addition to microbiome sequencing. In total, there will be 505 samples to be sequenced for microbiome composition and approximately 800 samples to be measured for digestive efficiency.

DNA Extraction and Amplification

Fecal DNA from frozen samples will be extracted using a ZYMO ZR-96 Fecal DNA Kit™ following the manufacturer's instructions (www.zymoresearch.com). PCR amplification of the 16S rDNA region will be performed with bar coded primers specific for the 16S rRNA region as previously described [68]. The 16s rRNA gene is found in all microorganisms and has enough sequence conservation for accurate alignment and enough variation for phylogenetic analyses.

To determine the possible contribution of bacteria from the diet on the mice's intestinal bacteria populations, representative food pellets have been selected to test the bacteria composition.

Microbiome Analysis

The 16S rDNA V4 region of the bacteria will be processed and sequenced using NextGen sequencing (MiSeq platform) by the UAB CCC/CFAR Microarray core (Comprehensive Cancer Center, Center for AIDS Research). The amplicons will be sequenced at 250 bp. Taxonomy of the gut microbiome sequences is assigned to the representative sequence of each operational taxonomic unit (OTU) using QIIME's parallel wrappers for the RDP classifier [69]. The most detailed taxonomic level assigned to an OTU's representative sequence at confidence of greater than or equal to 0.8 will be taken as the taxon of the OTU. The proportion of each taxon will be calculated as the proportion of each probe signal compared to the total signal.

Digestive Efficiency

Fecal samples for the digestive efficiency assay are collected from mice at the second weight fall of the WC groups (~ 20 months of age) for all groups. At the start of the collection, mice are placed in a clean cage with a measured amount of food and a cup of wood chip beddings. After 4 days, the food is reweighed, and the mice are given another clean cage. All fecal pellets will be collected and weighed for future analysis. Digestive efficiency will be assessed by comparing the calories consumed with the calories excreted in the feces. The feces collected will be dried to constant weight (at 60°C). The energy content of the food and the dried feces produced will be determined using a bomb calorimeter (Model 1261, Parr Instruments, Moline, IL). Digestive efficiency will be calculated as: (food energy ingested - fecal energy output) / (food energy ingested) *100%.

Statistical Analysis

The OTU data will be filtered before analyses because we do not want to consider those OTUs with low abundance, which may severely dilute the multiple testing power. Here are the criteria that we decided for the filtering at all levels: average absolute reads of OTUs are greater or equal to 20 (percentage wise, 0.01% ~ 0.1%); more than 50% samples under the same test have a read; and samples with total read counts are greater than 10,000.

Alpha diversity and beta diversity will be generated by QIIME [69]. Alpha diversity captures both the organismal richness (number) of a sample and the evenness (distribution) of the organism's abundance of distribution within a single population and is defined by the Chao1 index (richness, or the number) or the Shannon index (distribution, equitability or the evenness). Beta diversity represents the extent of similarity (or difference) in organismal composition between samples by measuring the degree to which membership or structure is shared between communities [70], as indicated by the Bray-Curtis dissimilarity (taxa overlap), weighted UniFrac or unweighted UniFrac (quantification of phylogenetic diversity) [71]. Sample similarities were projected onto two dimensions using principal coordinate analysis (PCoA).

Repeated measures analysis of variance (ANOVA) will be used to compare the body weight, body composition and food intake among groups over time. The Kruskal-Wallis or Mann-Whitney test will be used to identify statistically significant differences in microbial taxa, α diversity and other nonparametric measures. Spearman's correlation will be used to describe the relationship between gut microbiome composition and body weight and energy intake. UniFrac will be used to assess the overall differences among

the study groups by principal coordinates analysis. In addition, multivariate regression analysis will be used to explore associations between multiple exposure factors and microbiota composition. All analyses will be done using SAS 9.3 (SAS Institute Inc, NC) or R (www.r-project.org).

Terminology Disclaimer

Microbiome is defined as "ecological community of commensal, symbiotic and pathogenic microorganisms that literally share human body space" [72]. In the literature, microbiome and microbiota are often used synonymously. In this study, we would focus only on the bacteria in murine feces samples, but the terms microbiome, microbiota and microbes are also used interchangeably to represent "murine fecal bacteria."

Operational taxonomic unit is abbreviated as OTU and is defined as distinctive taxonomic level unit of sampling selected by the user to be used in a study, such as individuals, populations, species, genera or bacteria strains [73, 74]. Those categorized as the same OTU usually share a percent similarity threshold. In this dissertation, OTU is used to refer to the same bacterial category at various levels.

RESULTS AND DISCUSSION

Here I present the basic characteristics, followed by results and discussions, for each of the seven hypotheses. Only the data related to these mice included in this dissertation were included in this section.

Body weight, body composition and food intake of all time points are shown in **Figure 2**. Body compositions were scheduled to be measured at baseline and at each peak and trough of the WC group for all groups. Body weight and food intake were measured at the time of fecal sample collection for microbiome analysis and were obtained four times more from the WC group than from other groups due to the special interest in chronic changes of microbiome along with weight cycling.

Overall, these sub-samples followed the pattern of the original study in terms of body weight changes. Specifically, EO had gradual body weight increase with time as well as weight loss at later stages; OWLM and OWL had lower body weight than EO, respectively, and remained stable; WC group had two bouts of weight loss and regain cycles from baseline to the end; CULL group had a body weight lower than EO, despite continued *ad libitum* feeding. Fat mass resembles body weight in each of the groups, while the differences in fat-free mass are much smaller. The food intake was provisioned at certain amounts for OWL, OWLM and weight loss stages of the WC group. For EO, CULL and weight regain stages of the WC group, *ad libitum* feeding was provided. As seen in **Figure 2**, the food intake for the EO group increases and then gradually decreases as they age. The food intakes for OWL and OWLM were relatively stable because of

their targeted stable body weight. The food intake of the WC group was constantly adjusted to meet the designed body weight curves.

Overall, fecal samples from 507 samples (the majority of which were repeatedly selected) were measured by bar-coded pyrosequencing of the V4 region of 16S rDNA genes. After quality control of the sequencing results, there were a total of 45,735,335 OTU counts with an average of 90,928 counts per sample (±26,235 s.d.). From **Figure 3** we can see that there were great variations in both the Chao1 (A) and Shannon index (B), which represents richness (the number of bacteria) and evenness (equality of the distribution of bacterial groups), respectively. In the PCoA plots of β diversity by group*time, samples from different groups and time points were generally separated from each other, which suggest the effects of group and time on bacterial diversity in these samples. The Bray-Curtis β diversity (C, F, I) represents the dissimilarity among samples. UniFrac (weighted: D, G, J; unweighted: E, H, K) represents the phylogenetic diversity among samples.

The most abundant OTUs at the phylum level were *Firmicutes* (93.41%±5.52%), followed by *Actinobacteria* (2.96%±3.05%) and *Bacteroidetes* (2.73%±2.83%). Within the *Firmicutes* phylum, there were three abundant classes: *Clostridia* (57.25%±19.00%), *Erysipelotrichi* (28.84%±20.47%) and *Bacilli* (7.23%±7.73%). Most of the *Bacteroidetes* was accounted for by *Bacteroidia* class (2.73%±2.83%). Under *Actinobacteria* phylum, there were two major bacterial classes: *Actinobacteria* (1.71%±2.43%) and *Coriobacteriia* (1.25%±2.43%).

Figure 2. Basic characteristics for all mice: A. Body weight at fecal sample collection; B. Fat mass at each peak and trough; C. Fat-free mass at each peak and trough; D. Daily food intake around fecal sample collection. (n=10 for EO, 20 for OWL, OWLM and WC, 5 for CULL; week 44 was pre-randomization; all groups had the same type of high-fat diet.)

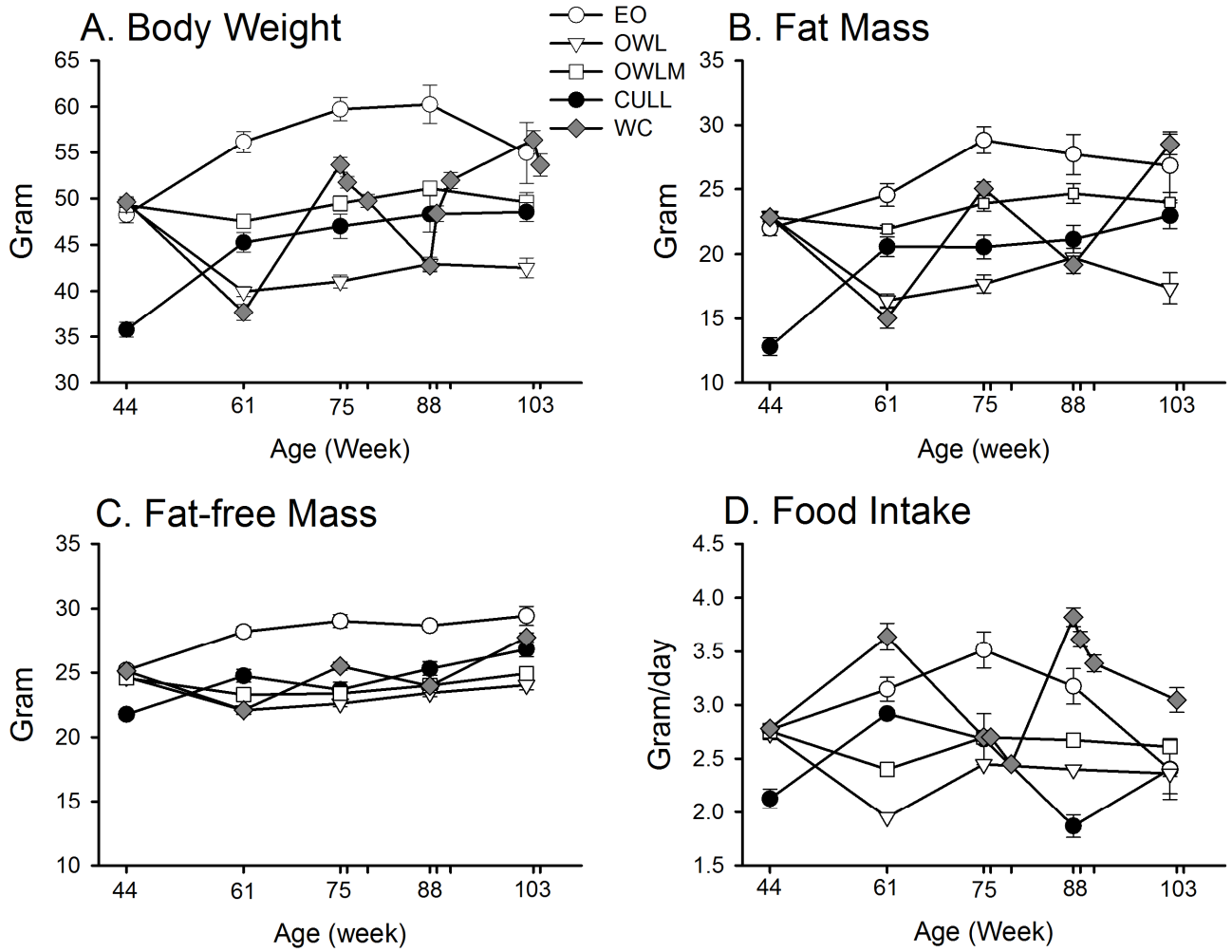
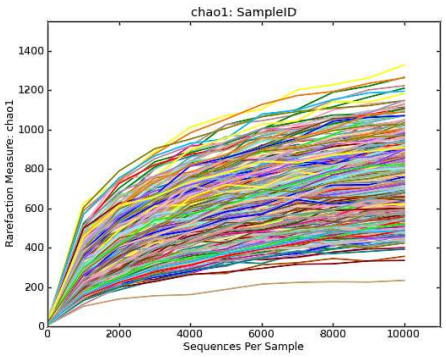


Figure 3. Diversity of the gut microbiota: A. Chao1 diversity for all samples; B. Shannon diversity for all samples; C.D.E.: beta diversity by group for all samples (Bray-Curtis, unweighted UniFrac, weighted UniFrac); F.G.H.: beta diversity by time point; I.J.K.: beta diversity by group*time point.

A.



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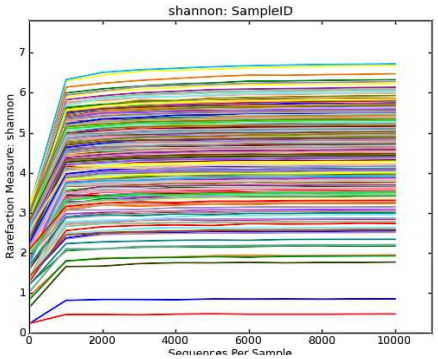
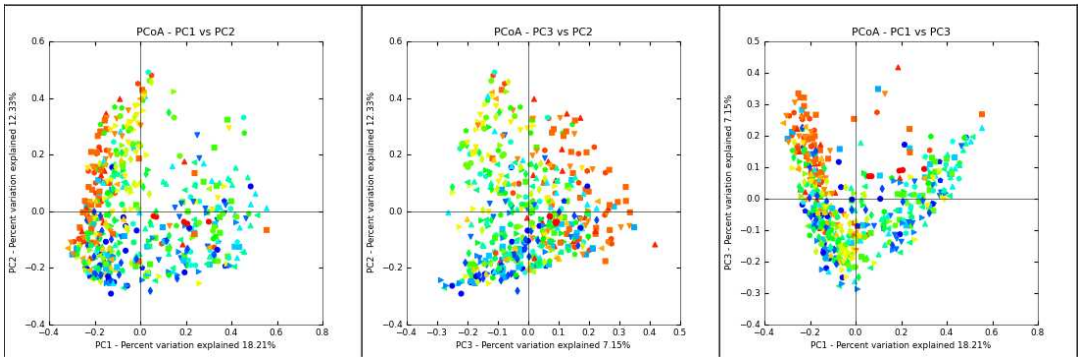
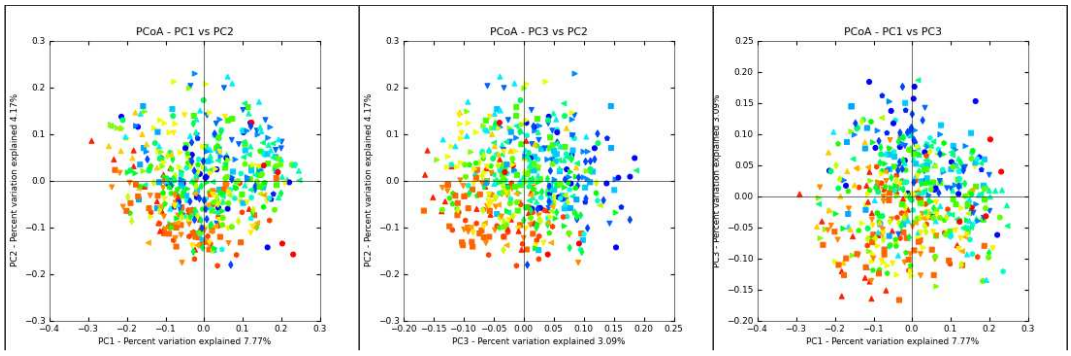


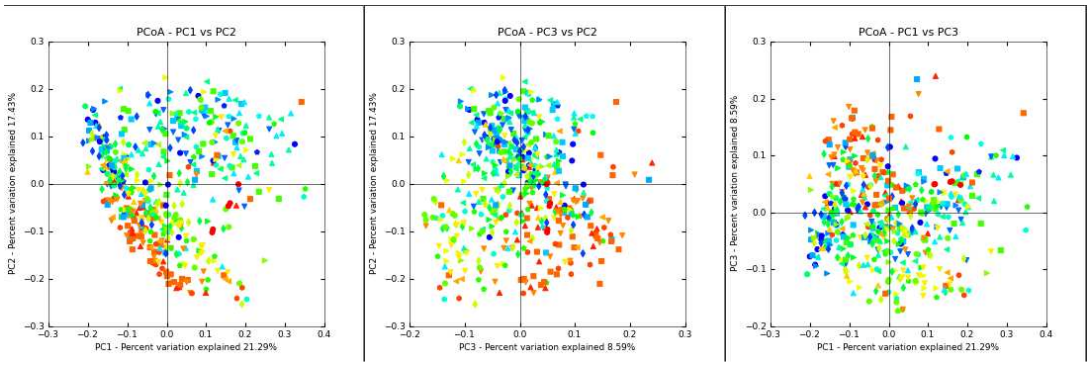
C. D. E.



F. G. H.



I. J. K.



This study has included robust sample sizes in testing the microbiome changes along with caloric restriction, weight cycling and aging in inbred C57BL/6J male mice. Because all of these mice were singly housed from 8 weeks of age under controlled environment and diets, we were able to minimize the influential factors such as cage-specific variations and inter-individual differences. To our knowledge, this is the first study to look at mice gut microbiome composition and its changes in different levels of calorie restriction and weight cycling, as well as longevity and aging. We showed that specific bacteria genera were different between high body weight and low body weight mice (both consuming *ad libitum* high-fat diet), related to digestive efficiency, enriched or decreased during calorie restriction, changed during long-term weight loss or regain, enriched during aging under fixed feeding regimen and, last, related to longevity.

First of all, of these ~500 hundred samples sequenced, there were great variability in α and β diversity. It is generally recognized that family members have a more similar community structure than unrelated individuals. While the animals in this study are an inbred strain, they still have individual variations in body weight, food intake and body composition under the same environment and feeding [75], which might be related to the variations and diversities in the microbial community or maternal influences. Different microbes metabolize dietary products in distinctive ways, but various diets also promote specific populations of microbial communities within the mother, the blood from which the fetus received could be substantially altered by the mother's microbes [76]. Contrary to the belief that the fetus develops within a sterile environment, recent evidence has shown that bacteria colonize the fetus before birth [77]. After birth, the microbial communities are influenced by interactions with mothers, other individuals as well as

environmental factors. A recent study suggests that host genotype has a measurable contribution to gut microbiome variation [78]. In mice, separating littermates into different cages (in this study all the mice were singly housed) can drive the differences in their microbiota further [79]. Unfortunately, we did not collect samples when the mice arrived, nor do we have maternal information.

Second, we found that the mice gut microbiome was dominated by *Firmicutes* phylum, which were much higher than previously reported in the same strain of mice [1, 27, 28, 32]. This could be caused by the diet differences, as the high-fat diet in this study was primarily composed of lard (45% kcal from fat, lard vs. soybean oil: 7.1:1 in weight), since diet itself [6] and the sources of dietary fat [80] could account for changes in gut microbiota compositions. For example, a low-fat diet was found to promote *Firmicutes* [81], but others found that a Western diet (typically composed of high fat) had higher abundance of *Firmicutes*, as well [28]. A greater amount of plant polysaccharides was found to be associated with low levels of *Firmicutes* and increased levels of *Bacteroidetes* [82]. In terms of fat source, milk-derived saturated fat resulted in a higher abundance of *Bacteroidetes* and a lower abundance of *Firmicutes*, while lard-based saturated fat has differential effects on phyla and sulfite-reducing bacteria compared to milk fat–based saturated fat or other fat sources [81]. Therefore, the abnormal high abundance of *Firmicutes* found in this study could be caused by the special fat composition (primarily lard) used in the high-fat diet.

Hypothesis 1a.

There Will Be No Differences between Fecal Bacteria Populations in Hosts with Significantly Different Levels of Adiposity under the Same Feeding Regimen

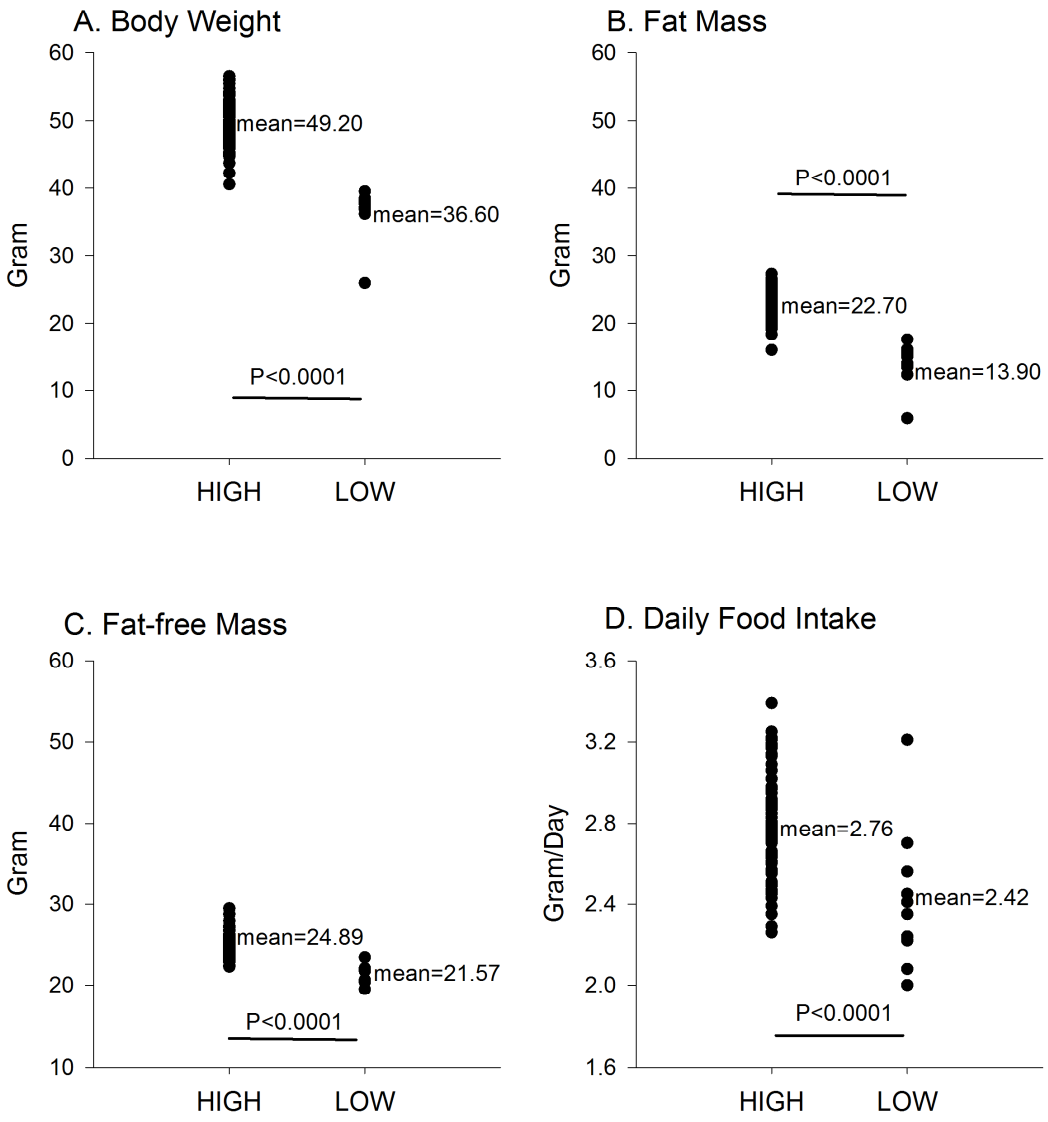
Results

Our previous study found that great variations existed in the body weight and fat mass in this strain of inbred C57BL/6J mice under *ad libitum* feeding with the same diet [75]. This chapter of the results tested the gut microbiome compositions related with body weight variations in two aspects: 1) the comparison of gut microbiome between the 100 high body weight mice and 10 low body weight mice before randomization, before which time point all mice had been receiving exactly same high-fat diet feeding; 2) association of specific gut microbiome species with body weight, fat mass, fat-free mass and daily food intake at the time of sample collection.

Figure 4 shows that there were significant differences (all $P<0.0001$) in body weight, fat mass and fat-free mass, as well as food intake, between the high body weight mice (n=100) and the low body weight mice (n=10). The body weight of high body weight mice ranged from 40 grams to almost 60 grams, while that of the low body weight mice was lower than 40 gram. A similar phenomenon was seen in fat mass and fat-free mass. However, the food intake of both groups had great variations, which failed to explain that the body weight difference was primarily caused by food intake.

At randomization, the high body weight mice were randomized into four different dietary treatment groups after this time point, and the low body weight mice were culled from the study because only those with diet-induced-obesity models would be included to test the effect of weight cycling and calorie restriction.

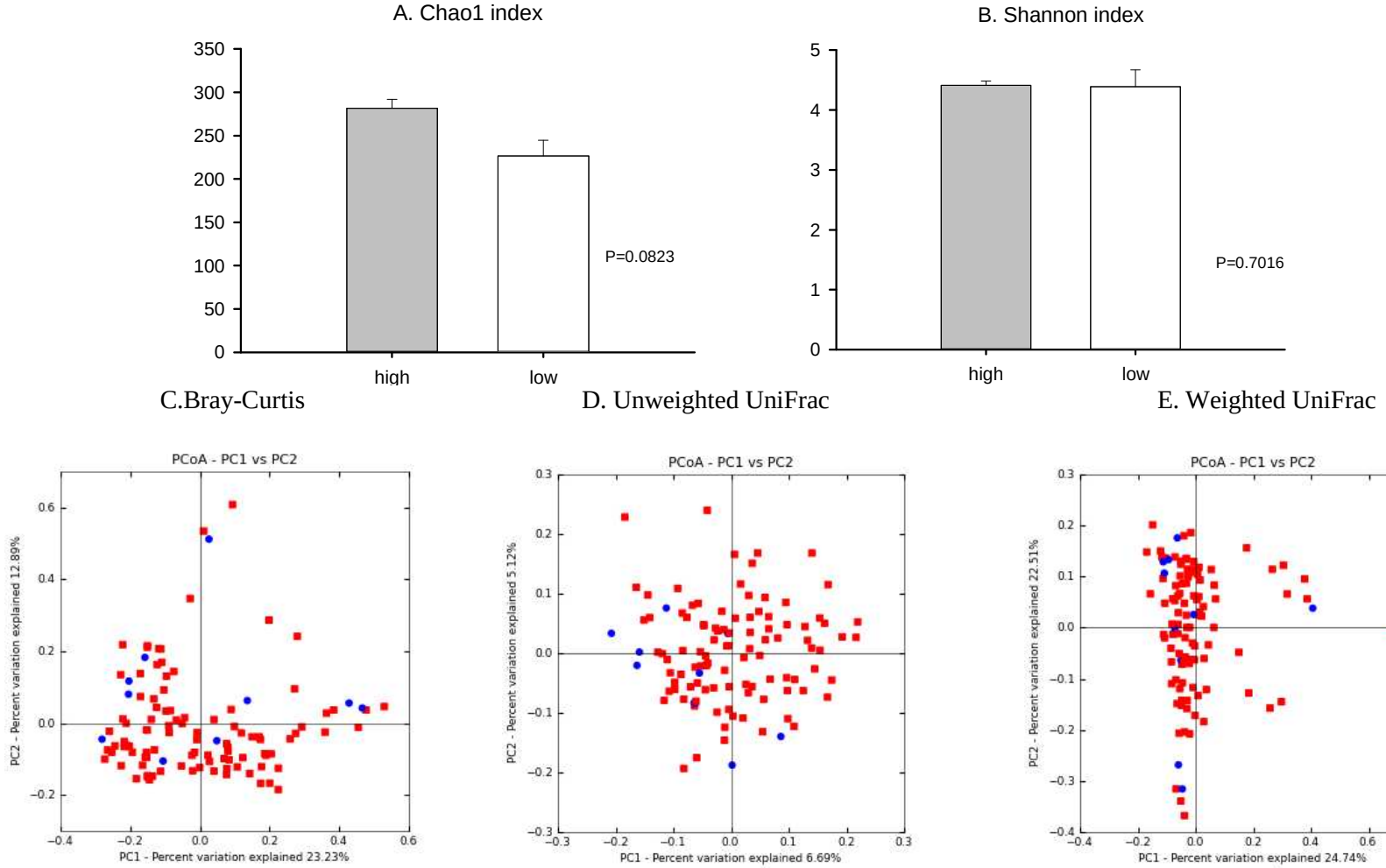
Figure 4. Body weight (A), fat mass (B), fat-free mass (C) and food intake (D) of the high body weight (n=100) and low body weight mice (n=10) before randomization at week 44. (Mean value of each parameter was shown next to the scatters; P-values were from the Wilcoxon-Mann-Whitney test.)



Alpha diversity within group richness and evenness (Chao1 index, Shannon Index, respectively) and principal-coordinates-based characterization (PCoA) of overall community structure (β diversity) for this hypothesis are shown in **Figure 5** below. As seen in the figure, there was no significant difference between these two groups in the Chao1 index score (P=0.0823, two sided, Mann-Whitney test) or the Shannon index score (P=0.7016), which indicates that the distributions of microbes were similar between the two body weight groups, and the trend is that the total number of microbes could be different if sample sizes are increased. Furthermore, PCoA analysis based on Bray-Curtis, weighted UniFrac or unweighted UniFrac did not show a clear clustering of groups.

To investigate the roles of specific gut microbiome in body weight variation, two regression models have been applied between microbiome abundance (%OTU) and bodyweight or food intake: 1a.1: %OTU = high body weight/low body weight; and 1a.2: %OTU = body weight + food intake. There were 149 OTUs (of these 110 samples) passed the filtering criteria (50% or more of the testing samples have an abundance of greater than 0 for each OTU; average reads greater or equal to 20 [~0.01%-0.1%]). After Bonferroni correction (0.05/149=0.0003), only three OTUs met the level of significance, and these three OTUs were in the same lineage from the family level: *Firmicutes* (phylum), *Bacilli* (class), *Lactobacillales* (order), *Streptococcaceae* (family) (estimate=21.32, std. error=2.83, t value=7.53, P=1.73E-11), Lactococcus (genus) (estimate=21.41, std. error=2.84, t value=7.54, P=61.1E-11) and unnamed (species) (estimate=21.43, std. error=2.84, t value=7.54, P=1.62E-11). These close values indicate that there was an unnamed species of bacteria under *Lactococcus* genus that was significantly different between the high body weight and low body mice. The average

Figure 5. Alpha and beta diversity for hypothesis 1a.1 (between the 100 high body weight and 10 low body weight mice at week 44): A. Chao1 index for richness (P=0.0823); B. Shannon Index for diversity (P=0.7016); C. Bray-Curtis beta diversity for dissimilarity; D. unweighted UniFrac beta diversity; E. weighted UniFrac beta diversity. In C–E, red represents the high body weight mice and blue the low body weight mice.



abundance of *Lactococcus* genus for the high body weight group was 0.67%±0.03%, and it was 2.29%±0.63% for the low body weight group.

In the second regression model of 1a.2, food intake has been adjusted to look at the associations between bacteria abundance and body weight. After Bonferroni correction (0.05/149=0.0003), the same three OTUs passed the test: *Streptococcaceae* (family) (estimate=-181.36, std. error=39.90, t value=-4.55, P=1.46E-05), *Lactococcus* (genus) (estimate=-182.23, std. error=40.00, t value=-4.56, P=1.369E-05) and unnamed (species) (estimate=-182.36, std. error=40.03, t value=-4.56, P=1.40E-05). These results suggest that the unnamed species of bacteria under *Lactococcus* genus was significantly negatively associated with body weight after adjusting for food intake. The average abundance of high body weight and low body weight combined was 0.82%±0.61%. Nevertheless, if the correlation model was applied to these two groups separately, there was no significant correlation between any of the OTUs and body weight or food intake. The Spearman correlation coefficient (rho) between *Lactococcus* genus and body weight was -0.25829 (P=0.0070). Notably, there were also several other OTUs that could be associated with body weight but didn't reach Bonferroni corrected P-value. These were: the *Clostridiaceae* family (estimate=12.01, P=0.0006, abundance=16.53%, correlation with body weight rho=0.27160, P=0.0045), unnamed genus and species under the *Clostridiaceae* family (estimate=12.43, P=0.0006), unnamed family/genus/species under the *Clostridiales* order (estimate=-24.04, P=0.0008), the *Clostriduium.celatum* species (estimate=1715.90, P=0.0051) and the *Oscillospira* genus (estimate=-33.69, P=0.006).

In addition, selected CULL (low body weight, n=5) and EO (high body weight, n=10) mice were compared (from 61 to 102 weeks of age) to observe the long-term

changes of gut microbiome between high body weight (EO) and low body weight (CULL) animals. The body weight and food intake of these two groups were shown in **Figure 6**. Although same strain of inbred mice under same life-long high-fat diet *ad libitum* feeding, the body weight ($P<0.0001$) and food intake ($P<0.0001$) were significantly different between the CULL and EO groups. With four time points combined, the alpha diversity (Chao1 and Shannon index, both $P<0.0001$) was significantly different between these two groups. The EO group had greater richness (Chao1 index) and evenness (Shannon index) in bacterial composition than CULL group (**Figure 7)**. In microbial community structure, these two groups were clearly separated from each other in the PCoA analysis between group diversity (Bray-Curtis, unweighted UniFrac and weighted UniFrac). If the two groups were viewed by different time points separately, there were significant differences in Chao1 at all time points ($P=0.0027$, 0.0059, 0.0027 and 0.0338, respectively), and the Shannon index was only different at time points 1 ($P=0.0059$) and 5 ($P=0.0027$), but not at time points 2 ($P=0.0576$) and 8 ($P=0.5101$).

Several statistical models were used to test the OTU differences between EO and CULL groups. Similar results were yielded between "%OTU=EO/CULL + time point" and "%OTU=EO/CULL + body weight + food intake." The first model looked at group differences controlling for time points, while the second model controlled for body weight, food intake and repeated effects. One genus of SMB53 under the *Clostridiaceae* family and the unnamed species under it were significantly different between the two groups ($P=2.79E-4$ for model1, $P=1.49E-4$). However, if the two models were merged into one, none of the OTUs was significantly different between the two groups.

Figure 6. Body weight and food intake of the obese-resistant (CULL) and obese-prone EO) C57BL/6J male mice: A. Body weight (mean±s.e.) from 8 weeks of age to 102 weeks of age; B. Average daily food intake (g/day, HFD, 45% cal from fat) at selected time points same as body weight. (Mice in both groups had *ad libitum* food intake across the study; weeks 8 to 43 were diet-induced obesity stage; weeks 67, 75, 88 and 102 coincided with the end of each weight loss or regain period for the WC group.)

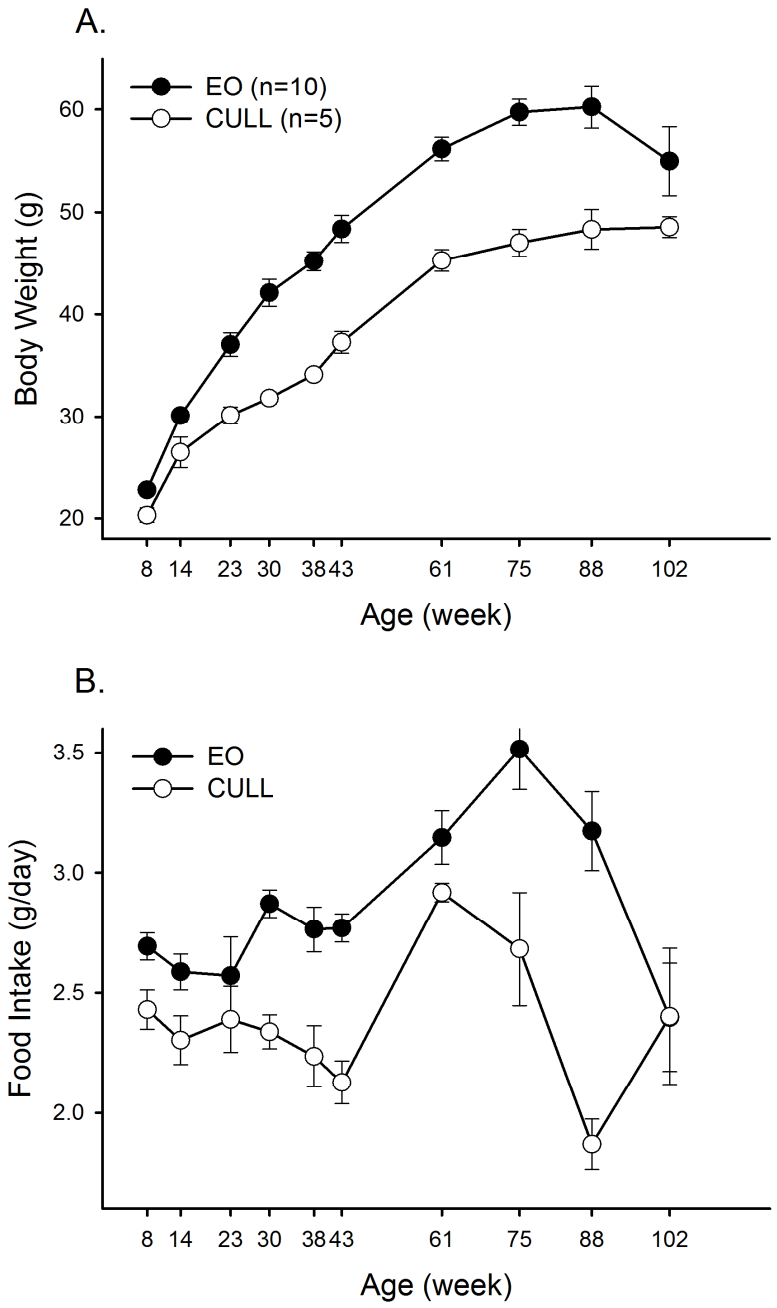
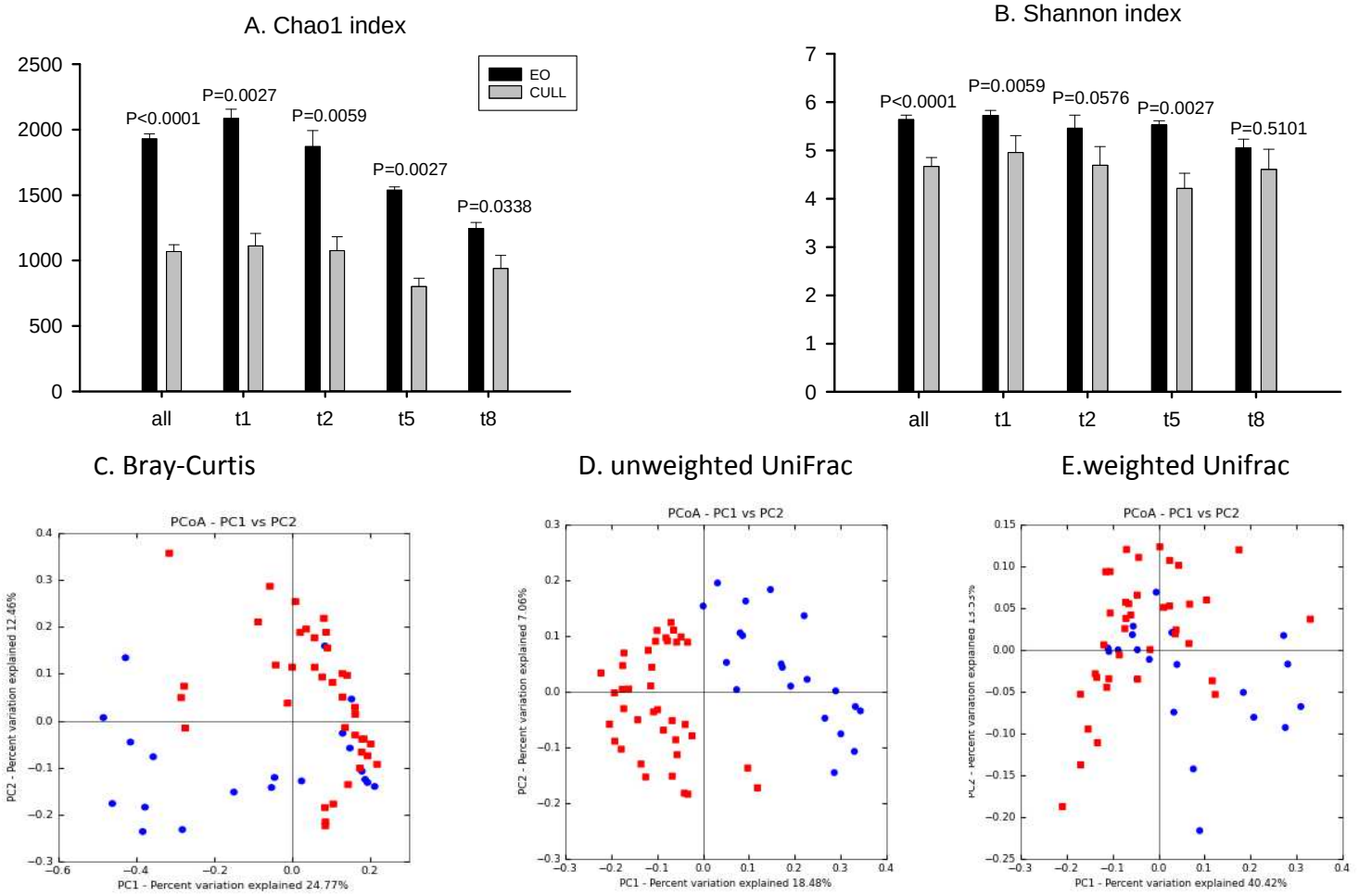


Figure 7. Alpha and beta diversity for hypothesis 1a.2. A. (obese-prone EO group/n=10 and obese-resistant CULL group/n=5; T1–week 61, T2–week 75, T5–week 88, T8–week 102, see Figure 6 for detail) Chao1 index for richness (P<0.0001); B. Shannon Index for diversity (P<0.0001); C. Bray-Curtis dissimilarity; D. unweighted UniFrac; E. weighted UniFrac. In C–E, red represents the EO group and blue the CULL group.



Discussion

Previous studies have found that obesity was associated with a reduction in alpha diversity [3]. We found that the richness (Chao1 index) and evenness (Shannon index) of bacteria were not significantly different between the high body weight and low body weight mice, which suggests that the overall bacterial number or evenness of distribution might not be associated with diet-induced obesity. Furthermore, the bacterial lineage of *Lactococcus* genus was found to be significantly different between high body weight (n=100) and low body weight (n=10) animals at randomization point. Additionally, after adjusting for food intake, this strain was significantly associated with body weight with the two groups combined but was not significant when the analysis was done separately for either of the groups. This suggests that the significant correlation between *Lactococcus* genus and body weight of the two groups combined was possibly caused by distinct higher abundance in the low body weight group. The genus *Lactococcus* is one of the lactic acid–producing bacteria [83], which are integral components of fermented food, where they produce lactic acid from glucose fermentation. *Lactococcus* includes seven different species. Some of these species are involved in technological food processing, while some can acquire antibiotic resistance under selective pressure [84]. An earlier study showed that high-fat–fed mice showed increased *Lactococcus* proportion (0.6%±0.1%) compared to low-fat–fed controls (0.4%±0.1%) [85]. While in this study, we found a similar proportion of *Lactococcus* in the high body weight mice (0.68%±0.03%); however, the low body weight mice under the same high-fat diet had significantly higher abundance (2.31%±0.63%). This matches another study that found a higher abundance of the *Streptococcaceae* family (which includes the *Lactococcus*

genus) in cecum samples from lean minipigs compared to obese minipigs under the same diet [86]. *Lactococcus* was also found to be positively correlated with gene expression levels in the inguinal fat of inflammation markers (*Saa3* and *Pai1*) [87]. These evidences suggest that both the diets and body weight could influence *Lactococcus* abundance, and that the abundance of *Lactococcus* could be associated with the development of obesity and underlying gene expression in fat.

The ratio of *Firmicutes:Bacteroidetes* was once proposed to be related to obesity [2], but we were unable to observe that between the high body weight (*Firmicutes:Bacteroidetes*=21.93) and low body weight mice (20.98) due to the abnormal high abundance of *Firmicutes*. This is in agreement with other studies, which suggests that the link between obesity and the microbiota is likely to be more sophisticated than the simple phylum-level *Firmicutes:Bacteroidetes* ratio [3, 88].

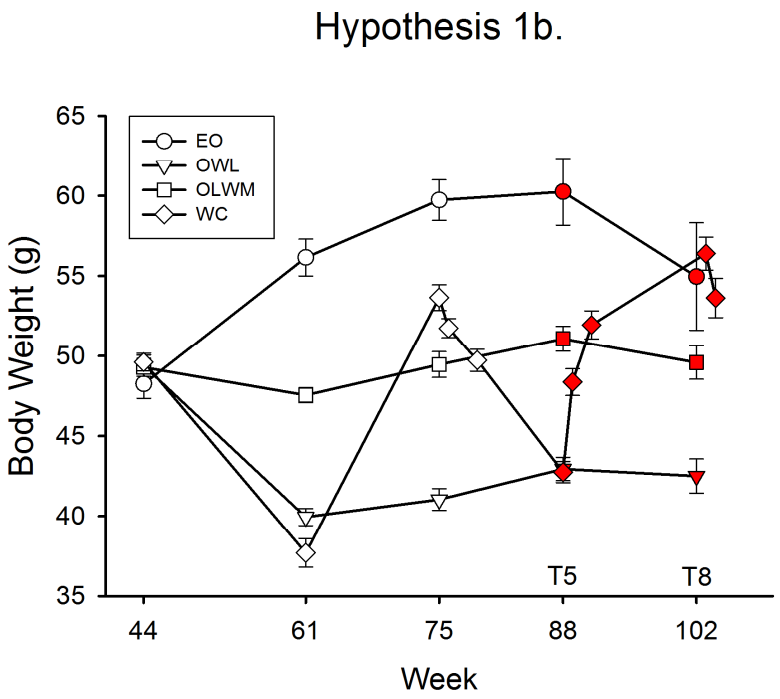
Contrary to the above, we also found that the richness (Chao1 index) and evenness (Shannon index) were significantly different between obese-prone (EO) and obese-resistant (CULL) groups. These two groups were also clearly separated from each other in the PCoA plots. This discrepancy might originate from selection bias because these samples were not randomly selected from all animals from the original mice longevity study. The results indicate that those obese-prone and obese-resistant mice under the same high-fat diet *ad libitum* feeding had dissimilarity in gut microbiome communities and that there were phylogenetic separations between the two groups. This could be caused by the differences in the amounts of food intake, which could be further tested below in the comparisons between EO and caloric restricted groups.

Overall, although all the mice in this hypothesis had received the same high-fat diet feeding and were singly housed under the same environment, there still were significant differences in certain bacteria, such as *Lactococcus*. Whether these differences are the cause or consequence of the variation in body weight is still unknown and needs further investigation with larger sample sizes.

Hypothesis 1b.

Fecal Bacteria Compositions (or Specific Strains of Bacteria) Are Not Associated with Digestive Efficiency (DE) or Fecal Energy Density

Figure 8. Average body weight for samples in hypothesis 1b (highlighted in red).



Results

A frequently proposed mechanism of gut microbiome–causing obesity is that certain types of intestinal bacteria are related to metabolism and energy conversion of

normally unabsorbed food material by host, which subsequently contributes to the energy available to the host. Studies have found that ob/ob mice have significantly higher acetate and butyrate content in the cecal contents but less energy remaining in their feces relative to their lean littermates, along with increased capacity for energy harvest from the diet [1]. At week 88, when the WC group was in its body weight trough, there was no significant difference in body weight between WC and OWL (P=0.9999). There were significant differences between any other pairs in body weight (all P<0.0001). EO had significantly lower fecal energy density than OWL (P=0.0006) and WC (P=0.0366); there were no significant differences between any other two groups (all P>0.05). At week 103, when the WC group was on its body weight peak, there was no significant difference in body weight between EO and WC (P=0.9660), EO and OWLM (P=0.1203); there were significant differences in body weight between any other two pairs (OWL vs. OWLM: P=0.0006; OWL vs. WC: P=<0.0001, OWL vs. EO: P<0.0001; OWLM vs. WC: P=0.0011). For fecal energy density, the *ad libitum*–fed EO group had significantly lower fecal energy density than other groups with lower body weight (P=0.0014 for OWL, P=0.0019 for OWLM) (**Figure 9.A and 9.B**). Similarly, WC had significantly lower fecal energy density than OWL (P<0.0001) and OWLM (P=0.0001). Digestive efficiency is negatively proportional to fecal energy density; consequently, the EO group should have higher digestive efficiency than other restricted groups. However, there were no significant differences among any groups at week 88 in digestive efficiency. At week 103, EO had significantly higher digestive efficiency than OWL (P=0.0002) and OWLM (P=0.0384); WC also had significantly higher digestive efficiency than OWL (P<0.0001)

and OWLM (P=0.0003); there was no significant difference between EO and WC (P=0.9819).

Within the WC group only, at first there was a significant increase in fecal energy density between weeks 88 and 89 (P=0.0046), but after that, fecal energy density kept decreasing, despite the stable increase in body weight (P<0.0001), until week 103 (**Figure 9.C**). From weeks 103 to 104, when the WC group went through one week of restriction after the body weight plateau, the fecal energy density was decreasing (P=0.0241). For digestive efficiency (**Figure 9.D**), week 89 had higher but not significantly (P=0.3151) different digestive efficiency than week 88, and the significant increase occurred since week 91 (P=0.0033). From weeks 103 to 104, the digestive efficiency decreased, as well (not statistically significant, P=0.2059). These results showed fecal energy density and digestive efficiency changed with the changes in food intake (or body weight) in the WC group. As the body weight of the WC group went up from peak to trough, fecal energy density decreased to the same level of the EO group of similar body weight. The changes in digestive efficiency were exactly in accordance with the changes in body weight, which was caused by changes in food intake.

Figure 9. Fecal energy density and digestive efficiency: A. Fecal energy density and B. Digestive efficiency at time points 5 (week 88) and 8 (week 103) for all four groups; C. Fecal energy density and D. Digestive efficiency for the WC group only. (Different letters represent significant differences among groups; n=10 for EO, 20 for WC, OWL and OWLM; approximate body weight trend lines were shown in A&C; for detail, see Figure 2.)

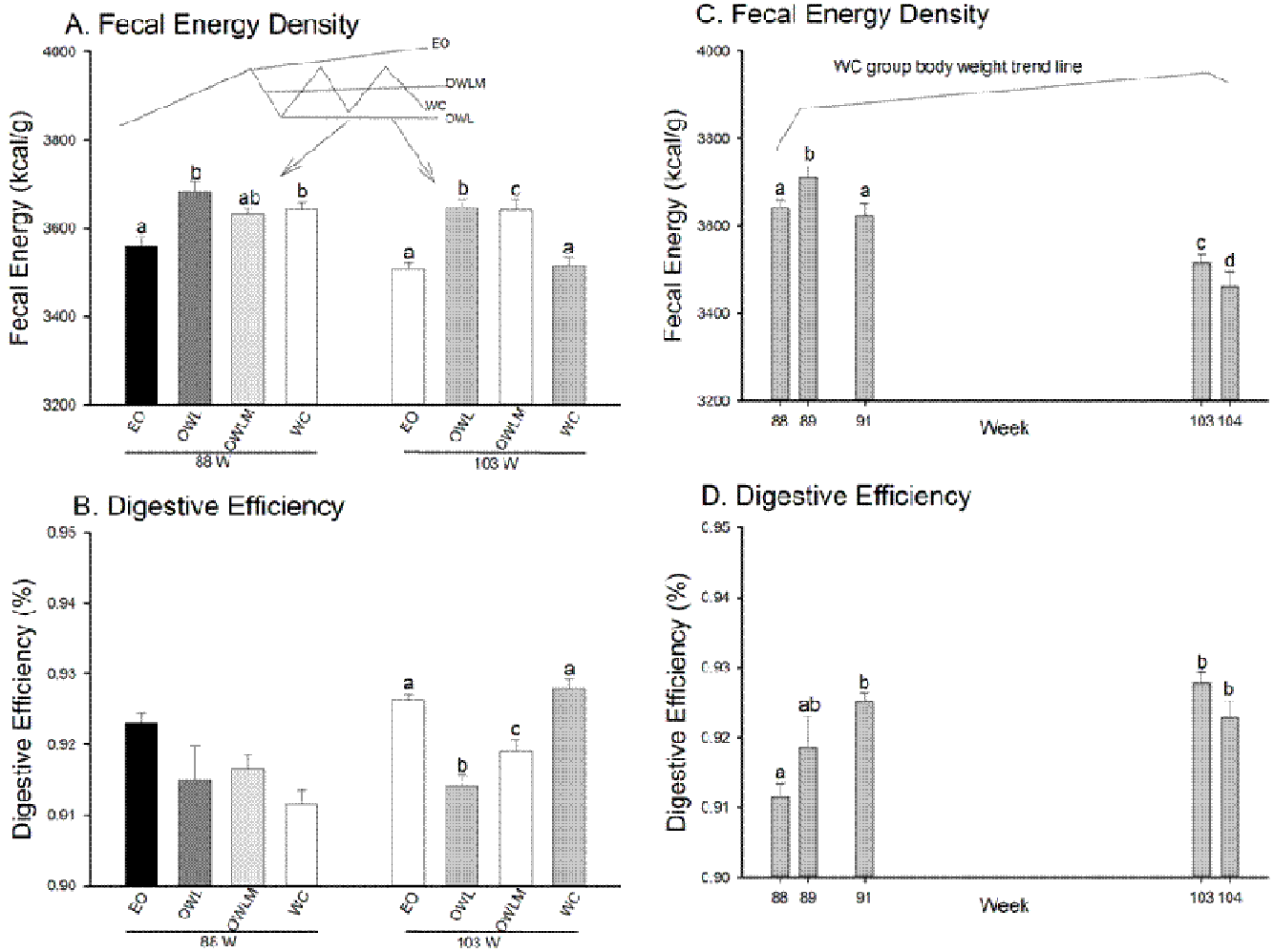


Figure 10 showed α and β diversity for the four experimental groups at time point 5 (age 88 weeks), when WC group was at its body weight trough. The EO group had both higher Chao1 ($P<0.0001$ with OWL and OWLM, $P=0.0002$ with WC) and Shannon ($P=0.0023$ with OWL, $P<0.0001$ with OWLM and WC) scores than the other three groups, indicating that this group had a greater number of OTUs, and they were more equally distributed within the group. There were no significant differences in Chao1 or the Shannon Index among WC, OWL or OWLM (all $P>0.05$). The unweighted UniFrac PCoA analysis showed that the community structure of EO (in red) and WC (in dark blue) clustered separately from the other two groups. Similarly, **Figure 11** showed α and β diversity for these groups at time point 8 (age 103 weeks), when the WC group regained the lost weight and was at its peak. At this time, there were only 8 animals left in the EO group. There were no significant differences in Chao1 ($P=0.2335$) or Shannon scores ($P=0.1942$) among these groups. Within the WC group, longitudinally, Chao1 score significantly increased from weeks 88 to 89 ($P=0.0275$), which was one week after *ad libitum* feeding, while the Shannon index remained unchanged ($P=0.2503$). From weeks 88 to 91 (three weeks after *ad libitum* feeding), neither the Chao1 ($P=0.3793$) nor the Shannon ($P=0.4407$) score changed significantly.

Figure 10. Alpha diversity (Chao1 and Shannon Index, mean±s.e.) and beta diversity (Bray-Curtis, unweighted UniFrac, weighted UniFrac) for time point 5 (week 88, second body weight trough point for WC group) for all four groups. For C–E: red – EO; light green – OWL; bright blue – OWLM; dark blue – WC.

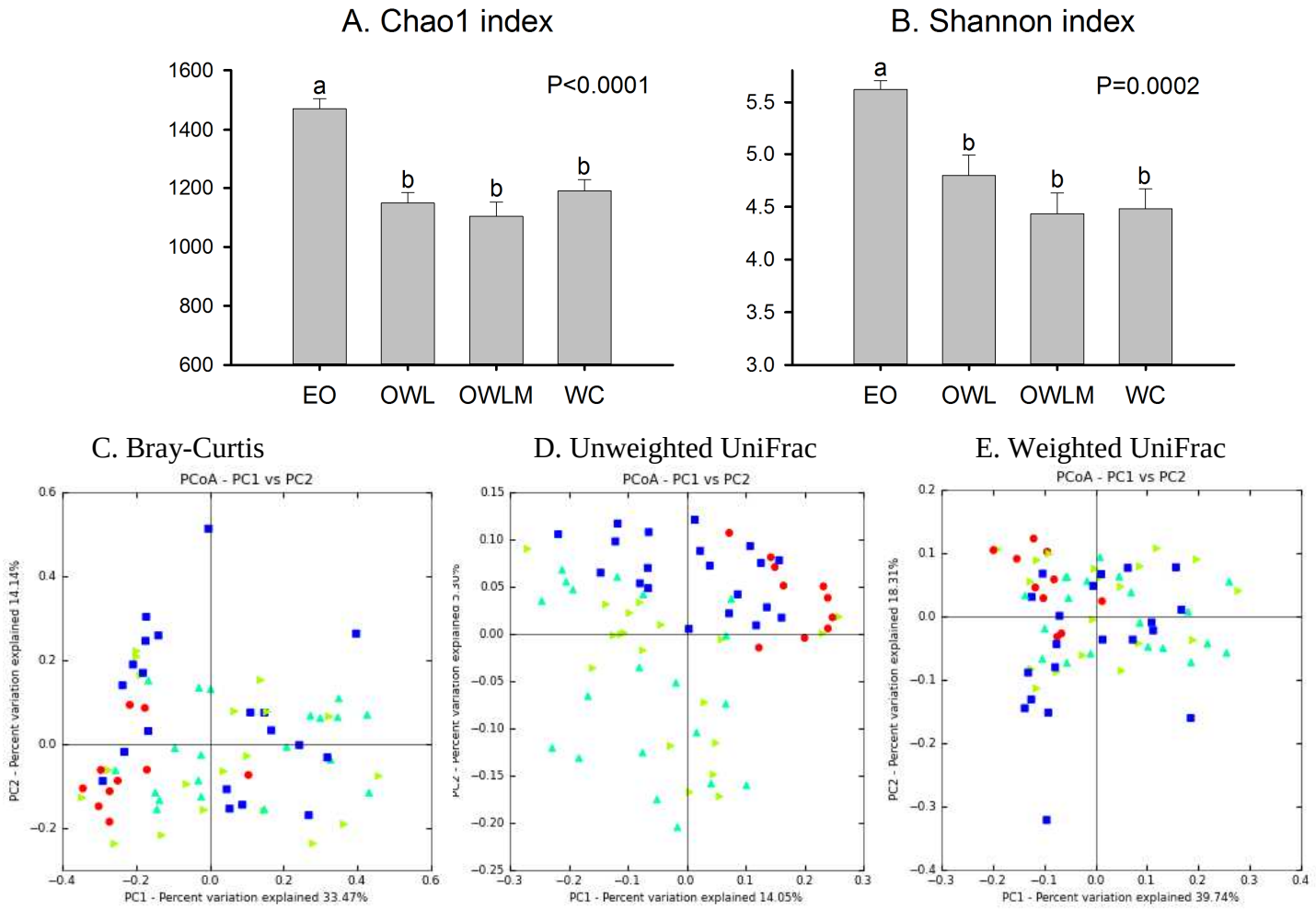
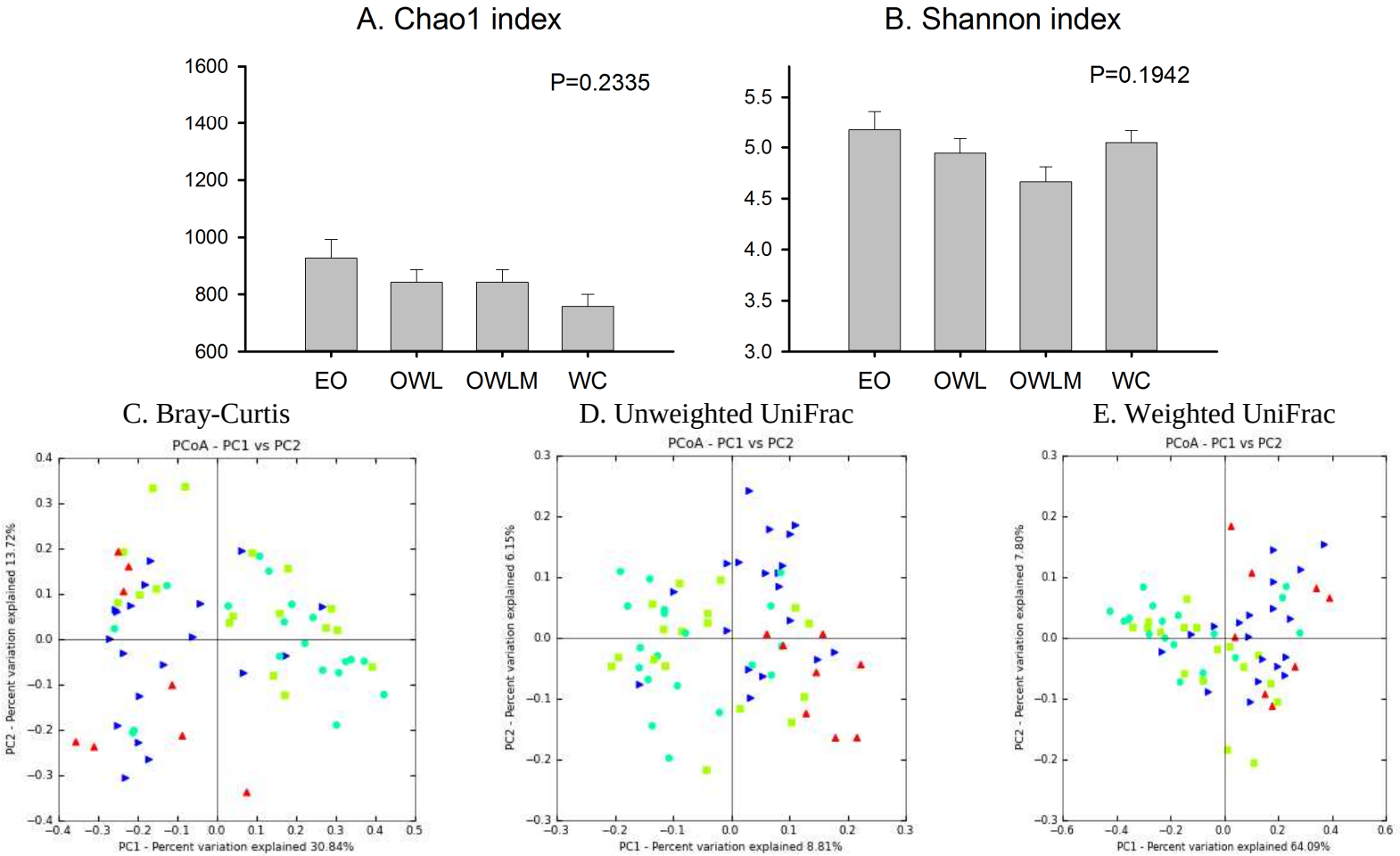


Figure 11. Alpha diversity (Chao1 and Shannon Index, mean±s.e.) and beta diversity (Bray-Curtis, unweighted UniFrac, weighted UniFrac) for time point 8 (week 103, second body weight peak for WC group) for all four groups. For C–E: red – EO; light green – OWL; bright blue – OWLM; dark blue – WC.



After filtering the OTU data by specific criteria (50% or more of the testing samples have an abundance of greater than 0 for each OTU; average reads greater or equal to 20 [~0.01%-0.1%]), a total of 149 OTUs were included in the statistical analysis. Multiple regression models have been used to explore the interactions among digestive efficiency, body weight, food intake, different groups and time points.

1b.1. To test the overall relationship between specific microbiome abundance (%OTU) and digestive efficiency (DE): %OTU = DE. After Bonferroni correction (0.05/144), about 25 OTUs at various levels showed significance (**Table 2**). From the table, we can see that all the significant hits were from *Firmicutes* at the phylum level, which indicates that, overall, the phylum *Firmicutes* was related to digestive efficiency. Furthermore, significant OTUs were at both higher and lower hierarchies, for example, from *Erysipelotrichi* (class) to *Allobaculum* (genus) (P=1.01E-07), which means that a specific group of bacteria at genus or species level has been detected to contribute to digestive efficiency in the bacterium lineage. Specifically, a species of bacterium under *Allobaculum* genus was responsible for the significance of *Erysipelotrichi* class. The estimate of *Allobaculum* genus (-7.49) in the model suggests these bacteria are negatively associated with digestive efficiency. *Clostridia* class and its lower hierarchies were also positively associated with digestive efficiency, with the *Clostridiales* order having the strongest effect. Moreover, the *Anaerovorax* genus (P=1.23E-04), the *Ruminococcus.gnavus* species (P=3.05E-04), the *Clostridiaceae* family (P=1.48E-04) and some unidentified bacterium under the *Bacilli* class (P=1.01E-01) were also found to be significantly correlated with digestive efficiency. Except for *Allobaculum*, the abundance of all other bacteria was positively correlated with digestive efficiency. Of them, there are

two very high-abundant bacterial classes: *Erysipelotrichi* (30.56%±1.59%) and C*lostridiales* class (58.52%±1.51%). Certain bacteria under *Bacilli* class were also significantly correlated with digestive efficiency, but they accounted for less than 4% of the total OTUs here in the table. Some other lower abundant bacteria, such as *Anaerovorax* genus and *Ruminococcus.gnavus*, are under the *Clostridiales* class.

1b.2. To test the interaction among gut microbiome abundance, digestive efficiency and food intake, as well as body weight: %OTU= DE + FI + BW. After Bonferroni correction, nine OTUs at various levels passed the significance test (**Table 3**). Of these nine OTUs, five shared the same values of estimate (-6.61), std. error (1.40), t value (-4.73) and P (4.54E-06). These five belong to the same bacterial line: *Firmicutes* (phylum), *Erysipelotrichi* (class), *Erysipelotrichaceae* (family), *Allobaculum* (genus) and unnamed (species), which indicates the unnamed species under *Allobaculum* was primarily responsible for the statistical significance in the correlation. The other four were from the *Clostridia* class (estimate=5.73, P= 4.13E-05), the *Clostridiales* order (estimate=5.73, P= 4.13E-05), the *Anaerovorax* genus (estimate=0.007, P=7.67E-05) and an unnamed species under the *Anaerovorax* genus (estimate=0.007, P=7.67E-05). Adding variables of food intake and body weight in the model decreased the total number of OTUs that showed significance, which suggests that the correlations between OTUs and digestive efficiency were partially explained by the correlations between OTUs and food intake or body weight. Interestingly, even after the adjustment of food intake and body weight, *Allobaculum* and *Anaerovorax* still remained significant. This indicates that the effects of these two genus-level bacteria on digestive efficiency are independent of food intake and body weight.

Table 2. Significant OTUs for regression model 1b.1 (model: %OTU=DE, P-value cutoff: 0.0003).

OTUs	Mean	s.e.	Estimate	s.e.	t value	Pr(>|t|)
k_Bacteria.p_Firmicutes.c_Erysipelotrichi	30.56%	1.59%	-7.49214	1.351386	-5.54404	1.01E-07
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales	30.56%	1.59%	-7.49214	1.351386	-5.54404	1.01E-07
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae	30.56%	1.59%	-7.49214	1.351386	-5.54404	1.01E-07
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum	30.19%	1.59%	-7.47699	1.349114	-5.54215	1.02E-07
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum.s_	30.19%	1.59%	-7.47699	1.349114	-5.54215	1.02E-07
k_Bacteria.p_Firmicutes.c_Clostridia	58.52%	1.51%	6.303591	1.306416	4.825103	2.92E-06
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales	58.52%	1.51%	6.303591	1.306416	4.825103	2.92E-06
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.02%	0.00%	0.007253	0.001825	3.973913	0.000101
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.02%	0.00%	0.007253	0.001825	3.973913	0.000101
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.02%	0.00%	0.007253	0.001825	3.973913	0.000101
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.02%	0.00%	0.007253	0.001825	3.973913	0.000101
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales	3.93%	0.41%	1.450112	0.367177	3.949352	0.000111
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae	3.93%	0.41%	1.450112	0.367177	3.949352	0.000111
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter	3.93%	0.41%	1.450112	0.367177	3.949352	0.000111
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter.s_	3.93%	0.41%	1.450112	0.367177	3.949352	0.000111
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_Anaerovorax	0.04%	0.00%	0.006015	0.001533	3.922512	0.000123
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_Anaerovorax.s_	0.04%	0.00%	0.006015	0.001533	3.922512	0.000123
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.Other	5.39%	0.49%	1.65419	0.426918	3.874723	0.000148
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.Other.Other	5.39%	0.49%	1.65419	0.426918	3.874723	0.000148
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae	5.65%	0.50%	1.694054	0.437961	3.868049	0.000152
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_.Ruminococcus..s_gnavus	1.62%	0.07%	0.21667	0.058864	3.680881	0.000305
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_.s_	5.27%	0.16%	0.216112	0.05872	3.680405	0.000305

Note: DE – digestive efficiency. Significant P values highlighted in bold.

Table 3. Significant OTUs for regression model 1b.2 (model: %OTU=DE+BW+FI; P-value cutoff: 0.0003).

OTUs	mean	s.e.	DE	BW	FI
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum	30.19%	1.60%	**4.54E-06**	0.0453	0.00681
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum.s_	30.19%	1.60%	**4.54E-06**	0.0453	0.00681
k_Bacteria.p_Firmicutes.c_Erysipelotrichi	30.56%	1.61%	**4.67E-06**	0.04324	0.00733
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales	30.56%	1.61%	**4.67E-06**	0.04324	0.00733
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae	30.56%	1.61%	**4.67E-06**	0.04324	0.00733
k_Bacteria.p_Firmicutes.c_Clostridia	58.52%	1.54%	**4.13E-05**	0.15797	0.0101
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales	58.52%	1.54%	**4.13E-05**	0.15797	0.0101
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_Anaerovorax	0.04%	0.00%	**7.67E-05**	0.39464	0.06095
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_Anaerovorax.s_	0.04%	0.00%	**7.67E-05**	0.39464	0.06095
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales	3.93%	0.27%	0.012798394	**8.48E-05**	0.49001
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae	3.93%	0.27%	0.012798394	**8.48E-05**	0.49001
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter	3.93%	0.27%	0.012798394	**8.48E-05**	0.49001
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter.s_	3.93%	0.27%	0.012798394	**8.48E-05**	0.49001
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.02%	0.00%	0.013394823	**4.48E-05**	0.45654
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.02%	0.00%	0.013394823	**4.48E-05**	0.45654
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.02%	0.00%	0.013394823	**4.48E-05**	0.45654
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.02%	0.00%	0.013394823	**4.48E-05**	0.45654

Note: DE – digestive efficiency; BW – body weight; FI – food intake. Significant P values highlighted in bold.

Table 4. Significant OTUs for regression model 1b.2a (model: %OTU=BW+FI; P-value cutoff: 0.0003).

OTUs	mean	s.e.	BW	FI
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.02%	0.00%	**3.55E-07**	0.478653
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.02%	0.00%	**3.55E-07**	0.478653
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.02%	0.00%	**3.55E-07**	0.478653
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.02%	0.00%	**3.55E-07**	0.478653
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales	3.93%	0.27%	**8.35E-07**	0.532401
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae	3.93%	0.27%	**8.35E-07**	0.532401
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter	3.93%	0.27%	**8.35E-07**	0.532401
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter.s_	3.93%	0.27%	**8.35E-07**	0.532401
k_Bacteria.p_Firmicutes.c_Bacilli	5.21%	0.29%	**1.09E-05**	0.75103
k_Bacteria.p_Actinobacteria.c_Coriobacteriia	1.35%	0.13%	**5.18E-05**	0.086821
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales	1.35%	0.13%	**5.18E-05**	0.086821
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae	1.35%	0.13%	**5.18E-05**	0.086821
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_	1.12%	0.12%	**9.53E-05**	0.051696
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_.s_	1.12%	0.12%	**9.53E-05**	0.051696
k_Bacteria.p_Actinobacteria	2.62%	0.18%	**0.000136**	0.371689
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_	0.07%	0.01%	**0.000235**	0.644467
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_.s_	0.07%	0.01%	**0.000235**	0.644467
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other	0.55%	0.02%	**0.000286**	0.038828
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other.Other	0.55%	0.02%	**0.000286**	0.038828
k_Bacteria.p_Firmicutes.Other	0.08%	0.01%	0.98187	**6.77E-05**
k_Bacteria.p_Firmicutes.Other.Other	0.08%	0.01%	0.98187	**6.77E-05**
k_Bacteria.p_Firmicutes.Other.Other.Other	0.08%	0.01%	0.98187	**6.77E-05**
k_Bacteria.p_Firmicutes.Other.Other.Other.Other	0.08%	0.01%	0.98187	**6.77E-05**
k_Bacteria.p_Firmicutes.Other.Other.Other.Other.Other	0.08%	0.01%	0.98187	**6.77E-05**

Note: BW – body weight; FI – food intake. Significant P values highlighted in bold.

1b.2a. To test the interaction between gut microbiome abundance, food intake and body weight: %OTU = FI + BW. Compared to 1b.2, this model removed the dependent variable of digestive efficiency to further test the relationship between OTU abundances and body weight/food intake (**Table 4**). Four bacterial lineages with low abundances were significantly correlated with body weight: unidentified species under the *Bacilli* class, the *Turicibacter* genus under the *Bacilli* class, the *Coriobacteriaceae* family under the *Actinobacteria* phylum, the *Clostridiaceae* family and the *Ruminococcaceae* family under the *Clostridia* class. One bacterial lineage was significantly associated with food intake: an unidentified species under *Firmicutes*. All of these bacterial lineages had lower abundances (from 0.02% to 5.21%).

1b.3. To test the interaction among microbiome abundance, digestive efficiency, food intake, body weight and group (EO as control, OWL, OWLM and WC as contrasts) at time point 5 (week 88, the body weight trough of WC group): %OTU = DE + FI + BW + group (results shown in **Table 5**). There were 70 samples in this model: 10 for EO and 20 for the other three groups each. After Bonferroni correction, there were no significant differences among any of the OTUs and digestive efficiency. However, one lineage of bacterium reached significance between OTU abundance and body weight (all levels of P=3.35E-04): *Verrucomicrobia* (phylum), *Verrucomicrobiae* (class), *Verrucomicrobiales* (order), *Verrucomicrobiaceae* (family), *Akkermansia* (genus) and *muciniphila* (species). Consequently, the other three groups were significantly different compared to the EO group, OWL (P=3.06E-07), OWLM (P=8.50E-07) and WC (P=2.67E-04), and these significances remained the same for all levels in this *Verrucomicrobia* lineage.

1b.4. To test the interaction among microbiome abundance, digestive efficiency, food intake, body weight and group at time point 8 (week 103, the body weight peak of WC group): %OTU = DE + FI + BW + group (results shown in **Table 6**). There were 70 samples in this model. Although this model was for the same animals but at a different time point from 1b.3, the bacteria that passed significance were different. After Bonferroni correction, one genus (and the species below) of bacterium under *Firmicutes* (phylum), *Clostridia* (class), *Clostridiales* (order) and *Clostridiaceae* (family) showed significant differences, after adjusting for digestive efficiency, body weight and food intake, between EO and three other groups: OWL (P=4.77E-5), OWLM (P=3.53E-07) and WC (P=2.00E-06). *Clostridium* (genus) of the same *Clostdiaceae* family was also significant (P=2.18E-04), but only between OWLM and EO. A bacterial lineage under *Firmicutes*, *Bacilli* (class) remained significant between these three groups (P=7.09E-05 for OWL, P=3.48E-06 for OWLM and P=2.64E-05 for WC) and EO. Another bacterial lineage was significantly different between the EO and OWLM groups: *Actinobacteria* (phylum, P=1.7E-04), *Coriobacteriia* (class, P=0.0001), *Coriobacteriales* (order, P=0.0001), *Coriobacteriaceae* (family, P=0.0001), unnamed genus (P=0.000277) and species (P=0.000277). Furthermore, *Adlercreutzia* genus and the unnamed species (under *Coriobacteriaceae* family) below were significantly different between groups OWL and EO (p=0.000244). Both *Clostridium* (genus) and *Clostridium.methylpentosum* (the *Firmicutes* phylum, the *Clostridia* class, the *Clostridiales* order and the *Ruminococcaceae* family) were significantly different between WC and EO (P=0.000262).

1b.5. This model combines 1b.3 and 1b.4 and controls the effect of time point and animal ID: %OTU = DE + FI + BW + group + time point + animal ID (results shown in **Table 7**). After controlling for body weight, food intake, group and time point, two bacterial lineages under *Firmicutes* phylum showed significance between %OTU and digestive efficiency: *Erysipelotrichi* (class), *Erysipelotrichales* (order), *Erysipelotrichaceae* (family), *Allobaculum* (genus) and unnamed species (all P=7.20E-07); *Clostridia* (class) and *Clostridiales* (order) (both P=2.35E-05). An unnamed bacterial lineage under *Bacilli* class from order to species also showed significance between %OTU and body weight (all P=4.42E-06), as well as %OTU and group (all P=2.69E-06). The *Turicibacterales* order, the *Turibacteraceae* family, the Turicibacter genus and an unnamed species under the *Bacilli* class all showed statistical significance between %OTU and body weight (all P=7.42E-05). The genus and species under the *Clostridiaceae* family, the *Clostridiales* order, the *Clostridia* class and the *Firmicutes* phylum both showed statistical significance between groups (both P=1.29E-04). The *Clostridium* genus and *C.ruminantium* under the *Peptostreptococcaceae* family, the *Clostridiales* order and the *Clostridia class* had statistical significances between groups (both P=3.42E-05). The SMB53 genus and an unnamed species below under the *Clostridiaceae* family also showed significant differences between groups (both P=8.71E-06).

1b.6 used only a paired t-test to investigate the digestive efficiency differences between time points 5 and 8 with all groups combined: DE = time point + animal ID and found that time point 8 has a significantly higher digestive efficiency than time point 5

(P=0.0048). This could be explained by the increase in digestive efficiency in the WC group.

1b.7 looked at the digestive efficiency differences between time points but controlled for food intake and body weight: DE = time point + FI + BW + animal ID. There were significant differences between time points after controlling for food intake and body weight (P=0.0042). Body weight was associated with digestive efficiency (P=0.0036), but food intake was not (P=0.9205).

1b.8 included only the WC group to test the relationships between bacteria abundance and digestive efficiency, food intake, body weight and time point: %OTU = DE + FI + BW + time point + animal ID (**Table 8**). *Erysipelotrichi* (class), *Erysipelotrichales* (order), *Erysipelotrichaceae* (family), *Allobaculum* (genus) and an unnamed species showed significant associations between %OTU and digestive efficiency (all P=3.93E-07), as well as %OTU and body weight (all P=1.1E-04). The *Clostridia* class and the *Clostridiales* order under *Firmicutes* also showed statistical significances in digestive efficiencies (both P=1E-05). A bacterial lineage of the *Actinobacteria* phylum (P=2.1E-05), the *Coriobacteriia* class (P=8.92E-05), the *Coriobacteriales* order (P=0.0000892), the *Coriobacteriaceae* family (P=8.92E-05) and an unnamed genus (P=5.42E-05) and species (P=5.42E-05) thereafter showed statistical significance in digestive efficiency. The unnamed genus and species under *Coriobacteriaceae* family also showed statistical significance in body weight (both P=1.36E-04). An unidentified genus and species under the *Firmicutes* phylum, the *Clostridia* class, the *Clostridiales* order and the *Lachnospiraceae* family showed statistical significance in digestive efficiency (both P=2.37E-04). Several other OTUs

under the *Clostridiales* order had marginal statistical significance in digestive efficiency, as well (with P-values slightly over Bonferroni corrected 0.0003).

1b.9 used the same regression model as 1b8 but removed the food intake variable to test the model without adjusting food intake: %OTU = DE + BW + time point + animal ID. Same as 1b.8, the bacterial lineage of *Allobaculum* showed statistical significance in digestive efficiency (all P=3.75E-07) and body weight (from the *Erysipelotrichi* class to the *Erysipelotrichaceae* family, all P=1.16E-04; *Allobaculum* and its subordinate species, both P=1.1E-04), as well as in time point (from the *Erysipelotrichi* class to the *Erysipelotrichaceae* family, all P=3.39E-04; *Allobaculum* and its subordinate species, both P=2.88E-04). The *Clostridia* class and the *Clostridiales* order (both P=1.04E-05) and an unidentified genus and species of *Lachnospiraceae* family (both P=2.16E-04) showed statistical significances in digestive efficiency. The *Actinobacteria* phylum (P=0.2E-05), the *Coriobacteriia* class (P=8.22E-05), the *Coriobacteriales* order (P=8.22E-05), the *Coriobacteriaceae* family (P=8.22E-05) and an unnamed subordinate genus and species (both P=4.96E-05) showed statistical significance in digestive efficiency. Of this *Actinobacteria* lineage, the unnamed genus and species (both P=1.2E-04) and the three OTUs above (from class to family, all P=2.88E-05) showed statistical significances in body weight, as well.

1b.10 removed body weight in the regression model of 1b.8: %OTU = DE + FI + time point + animal ID. Again, the bacterial lineage of *Allobaculum* (from class to species) showed statistical significance in digestive efficiency (all P=4.02E-07) and in food intake, as well (P=9.52E-06 from class to family, P=7.14E-06 for the genus and species). The *Clostridia* class and the *Clostridiales* order (both P=8.85E-06) and an

unidentified genus and species under *Lachnospiraceae* family (both P=2.34E-04) showed statistical significance in digestive efficiency. The *Actinobacteria* phylum (P=2.50E-05), the *Coriobacteriia* class (P=9.37E-05), the *Coriobacteriales* order (P=9.37E-05), the *Coriobacteriaceae* family (P=9.37E-05) and an unnamed genus and species (both P=5.88E-05) showed statistical significance in digestive efficiency, as well.

1b.11 removed both food intake and body weight from the model: %OTU = DE + time point + animal ID. Similar to the above, the bacterial lineage of *Allobaculum* remained significance in digestive efficiency (from class to family, P=3.25E-07; genus and species, P=3.83E-07). Since the variables of food intake and body weight were removed, time point showed significance in this model (from class to family, P=3.92E-07; genus and species, P=3.25E-07). The *Clostridia* class (P=9.19E-06) and the *Clostridiales* order (P=9.19E-06), as well as an unidentified genus and species (both P=0.000212) under *Lachnospiraceae* remained significant in digestive efficiency. The *Actinobacteria – Coriobacteriaceae* lineage also showed statistical significance in digestive efficiency (P=2.29E-05 for phylum, P=8.59E-05 for class, order and family, P=5.37E-05 for genus and species). Additionally, this lineage showed statistical differences between time points from the class level down (P=1.49E-04 for class, order and family, P=7.45E-05 for genus and species).

1b.12 removed the variables of digestive efficiency and body weight from the model: %OTU = FI + time point + animal ID. This model tested the OTU difference between time points adjusting for food intake. Interestingly, all of the significant OTUs were at class level or higher. The major phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Tenericutes* and *Verrucomicrobia* were significantly associated with food

intake. At the class level, *Actinobacteria*, *Coriobacteriia*, *Bacteroidia*, *Bacilli*, *Clostridia*, *Erysipelotrichi*, *Gammaproteobacteria*, *Mollicutes* and *Verrucomicrobiae* were significantly associated with food intake. However, these significant OTUs had a weak Spearman correlation coefficient. Take, for example, the Spearman correlation coefficient between food intake and the *Actinobacteria* phylum (rho=0.33317, P=0.0008) and the *Erysipelotrichi* phylum (rho=0.35316, P=0.0004).

1b.13. To test the relationship between body weight and digestive efficiency for OWLM and OWL group at time points 5 and 8, at which time the daily food intake was constant: BW=DE + time point. There was no statistical significance in digestive efficiency (P=0.134088) or time point (P=0.705682).

Table 5. Significant OTUs for regression model 1b.3 (model: %OTU = DE + FI + BW + group; P-value cutoff: 0.0003).

OTUs	mean	s.e.	DE	BW	FI	Group OWL	Group OWLM	Group WC
k_Bacteria.p_Verrucomicrobia	0.53%	0.10%	0.78265	**0.00014**	0.20271	**3.06E-07**	**8.50E-07**	**0.00027**
k_Bacteria.p_Verrucomicrobia.c_Verrucomicrobiae	0.53%	0.10%	0.78265	**0.00014**	0.20271	**3.06E-07**	**8.50E-07**	**0.00027**
k_Bacteria.p_Verrucomicrobia.c_Verrucomicrobiae.o_Verrucomicrobiales	0.53%	0.10%	0.78265	**0.00014**	0.20271	**3.06E-07**	**8.50E-07**	**0.00027**
k_Bacteria.p_Verrucomicrobia.c_Verrucomicrobiae.o_Verrucomicrobiales.f_Verrucomicrobiaceae	0.53%	0.10%	0.78265	**0.00014**	0.20271	**3.06E-07**	**8.50E-07**	**0.00027**
k_Bacteria.p_Verrucomicrobia.c_Verrucomicrobiae.o_Verrucomicrobiales.f_Verrucomicrobiaceae.g_Akkermansia	0.53%	0.10%	0.78265	**0.00014**	0.20271	**3.06E-07**	**8.50E-07**	**0.00027**
k_Bacteria.p_Verrucomicrobia.c_Verrucomicrobiae.o_Verrucomicrobiales.f_Verrucomicrobiaceae.g_Akkermansia.s_muciniphila	0.53%	0.10%	0.78265	**0.00014**	0.20271	**3.06E-07**	**8.50E-07**	**0.00027**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_	5.27%	0.14%	0.8693	0.08033	0.84944	0.00365	**0.00025**	0.00855
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_.Ruminococcus.	1.64%	0.07%	0.8693	0.08033	0.84944	0.00365	**0.00025**	0.00855
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae	0.04%	0.00%	0.03308	0.10408	0.40613	**0.0002**	**9.49E-07**	0.00778
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae.g_Dehalobacterium	0.04%	0.00%	0.03308	0.10408	0.40613	**0.0002**	**9.49E-07**	0.00778
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae.g_Dehalobacterium.s_	0.04%	0.00%	0.03308	0.10408	0.40613	**0.0002**	**9.49E-07**	0.00778
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae	12.63%	0.36%	0.08799	0.44809	0.56287	0.02937	**0.0003**	0.09161
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53	0.11%	0.01%	0.9633	0.61292	0.39637	0.01732	**0.00012**	0.00797
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53.s_	0.11%	0.01%	0.9633	0.61292	0.39637	0.01732	**0.00012**	0.00797

Note: DE – digestive efficiency, FI – food intake, BW – body weight. Significant P values highlighted in bold.

Table 6. Significant OTUs for regression model 1b.4 (model: %OTU = DE + FI + BW + group; P-value cutoff: 0.0003).

OTUs	mean	s.e.	DE	BW	FI	Group OWL	Group OWLM	Group WC
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_	0.07%	0.01%	0.857827	0.767609	0.076028	**4.77E-05**	**3.53E-07**	**2.00E-06**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_.s_	0.07%	0.01%	0.857827	0.767609	0.076028	**4.77E-05**	**3.53E-07**	**2.00E-06**
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.02%	0.00%	0.616153	0.350444	0.19104	**7.09E-05**	**3.48E-06**	**2.64E-05**
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.02%	0.00%	0.616153	0.350444	0.19104	**7.09E-05**	**3.48E-06**	**2.64E-05**
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.02%	0.00%	0.616153	0.350444	0.19104	**7.09E-05**	**3.48E-06**	**2.64E-05**
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.02%	0.00%	0.616153	0.350444	0.19104	**7.09E-05**	**3.48E-06**	**2.64E-05**
k_Bacteria.p_Actinobacteria.c_Coriobacteriia	1.35%	0.13%	0.243686	0.193473	0.406678	0.00256	**0.0001**	0.310353
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales	1.35%	0.13%	0.243686	0.193473	0.406678	0.00256	**0.0001**	0.310353
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae	1.35%	0.13%	0.243686	0.193473	0.406678	0.00256	**0.0001**	0.310353
k_Bacteria.p_Actinobacteria	2.62%	0.18%	0.262379	0.266245	0.55815	0.009176	**0.00017**	0.643635
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_Clostridium	0.08%	0.01%	0.845105	0.463861	0.313723	0.001262	**0.000218**	0.00091
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_	1.12%	0.12%	0.24389	0.198347	0.43255	0.006484	**0.000277**	0.471141
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_.s_	1.12%	0.12%	0.24389	0.198347	0.43255	0.006484	**0.000277**	0.471141
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia	0.22%	0.01%	0.904879	0.807216	0.583763	**0.000244**	0.00043	0.001312
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia.s_	0.22%	0.01%	0.904879	0.807216	0.583763	**0.000244**	0.00043	0.001312
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium	0.07%	0.00%	0.969094	0.929416	0.395366	0.305872	0.189119	**0.000262**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium.s_methylpentosum	0.07%	0.00%	0.969094	0.929416	0.395366	0.305872	0.189119	**0.000262**

Note: DE – digestive efficiency, FI – food intake, BW – body weight. Significant P values highlighted in bold.

Table 7. Significant OTUs for regression model 1b.5 (model: %OTU= DE + FI + BW + group + time point + animal ID; P-value cutoff: 0.0003).

OTUs	mean	s.e.	DE	BW	BW	Group	Time point
k_Bacteria.p_Firmicutes.c_Erysipelotrichi	30.56%	1.61%	**7.20E-07**	0.02108	0.10016	0.00188	0.01465
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales	30.56%	1.61%	**7.20E-07**	0.02108	0.10016	0.00188	0.01465
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae	30.56%	1.61%	**7.20E-07**	0.02108	0.10016	0.00188	0.01465
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum	30.19%	1.60%	**7.39E-07**	0.02184	0.09543	0.00196	0.01644
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum .s_	30.19%	1.60%	**7.39E-07**	0.02184	0.09543	0.00196	0.01644
k_Bacteria.p_Firmicutes.c_Clostridia	58.52%	1.54%	**2.35E-05**	0.14005	0.21132	0.00857	0.05305
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales	58.52%	1.54%	**2.35E-05**	0.14005	0.21132	0.00857	0.05305
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.02%	0.00%	0.00141	**4.42E-06**	0.77272	**2.69E-06**	0.87569
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.02%	0.00%	0.00141	**4.42E-06**	0.77272	**2.69E-06**	0.87569
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.02%	0.00%	0.00141	**4.42E-06**	0.77272	**2.69E-06**	0.87569
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.02%	0.00%	0.00141	**4.42E-06**	0.77272	**2.69E-06**	0.87569
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales	3.93%	0.27%	0.00421	**7.42E-05**	0.93012	0.13794	0.24133
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae	3.93%	0.27%	0.00421	**7.42E-05**	0.93012	0.13794	0.24133
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter	3.93%	0.27%	0.00421	**7.42E-05**	0.93012	0.13794	0.24133
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter.s_	3.93%	0.27%	0.00421	**7.42E-05**	0.93012	0.13794	0.24133
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_	0.07%	0.01%	0.01698	0.0015	0.67626	**0.00013**	0.13431
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_.s_	0.07%	0.01%	0.01698	0.0015	0.67626	**0.00013**	0.13431
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium	0.03%	0.00%	0.19995	0.93098	0.00639	**3.42E-05**	0.60274
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium.s_ruminantium	0.03%	0.00%	0.19995	0.93098	0.00639	**3.42E-05**	0.60274
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53	0.11%	0.01%	0.63704	0.13454	0.25287	**8.71E-06**	0.3801
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53.s_	0.11%	0.01%	0.63704	0.13454	0.25287	**8.71E-06**	0.3801

Note: DE – digestive efficiency, FI – food intake, BW – body weight. Significant P values highlighted in bold.

Table 8. Significant OTUs for regression model 1b.8 (model: %OTU= DE + FI + BW + group + time point + animal ID for WC only; P-value cutoff: 0.0003).

OTUs	mean	s.e.	DE	BW	FI	Time point
k_Bacteria.p_Firmicutes.c_Erysipelotrichi	30.56%	1.61%	**3.93E-07**	**0.00011**	0.00142	0.04708
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales	30.56%	1.61%	**3.93E-07**	**0.00011**	0.00142	0.04708
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae	30.56%	1.61%	**3.93E-07**	**0.00011**	0.00142	0.04708
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum	30.19%	1.60%	**4.58E-07**	**0.0001**	0.00113	0.04681
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum.s_	30.19%	1.60%	**4.58E-07**	**0.0001**	0.00113	0.04681
k_Bacteria.p_Firmicutes.c_Clostridia	58.52%	1.54%	**1.00E-05**	0.02304	0.01342	0.43307
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales	58.52%	1.54%	**1.00E-05**	0.02304	0.01342	0.43307
k_Bacteria.p_Actinobacteria	2.62%	0.18%	**2.10E-05**	0.00158	0.21242	0.88734
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_	1.12%	0.12%	**5.42E-05**	**0.00014**	0.12479	0.23039
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_.s_	1.12%	0.12%	**5.42E-05**	**0.00014**	0.12479	0.23039
k_Bacteria.p_Actinobacteria.c_Coriobacteriia	1.35%	0.13%	**8.92E-05**	0.00032	0.13889	0.2317
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales	1.35%	0.13%	**8.92E-05**	0.00032	0.13889	0.2317
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae	1.35%	0.13%	**8.92E-05**	0.00032	0.13889	0.2317
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.Other	3.58%	0.11%	**0.00024**	0.22912	0.38391	0.70912
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.Other.Other	3.58%	0.11%	**0.00024**	0.22912	0.38391	0.70912

Note: DE – digestive efficiency, FI – food intake, BW – body weight. Significant P values highlighted in bold.

Discussion

It was initially hypothesized that to compensate for reduced caloric intake (calorie restriction), animals may increase their digestive efficiency to extract more energy from the ingested food, but later study did not support this argument when no difference was found in digestive efficiency between controls and 20% restricted animals [89]. It was found that the changes in gut microbiota after nutrient load alteration were directly correlated with stool energy loss such that a 20% increase in Firmicutes and a corresponding decrease in Bacteroidetes were associated with an increased energy harvest of about 150 kcal [42]. Here in this study, for the first time, we tested the energy outputs of feces for animals that had undergone repeated weight loss and regain cycles, in addition to two different levels of calorie restriction, which shall provide adequate evidence to test the relationships among calorie restriction, digestive efficiency and gut microbiome composition.

At week 88 (~40 weeks of calorie restriction), we found that there was a significant difference between the OWL group (~25% restriction) and the EO group, with the OWL group having higher energy content per gram of dry feces than the EO group. Although not statistically significant, EO had, on average, lower fecal energy content than OWLM and then WC, which suggests an inverse body weight–dependent relationship. Since digestive efficiency is inversely associated with fecal energy content, the digestive efficiency was highest in the *ad libitum*–fed EO group, lower in the OWLM group and lowest in the OWL group. However, there were no significant differences in digestive efficiency between any of the two groups. At week 103, which was at about 60 weeks of calorie restriction for the OWLM and OWL groups, or two bouts of weight loss

and regain cycles for the WC group, the WC group was at its body weight peak, which was close to the body weight of the EO group. The EO and WC groups had significantly lower fecal energy density and significantly higher digestive efficiency than OWL and OWLM. The information above suggests that fecal energy content is inversely related to food intake, and digestive efficiency is positively related to food intake.

For the relationships between OTU abundance and digestive efficiency, two bacterial lineages with high abundances were associated with digestive efficiency overall: the *Allobaculum* genus and the *Clostridiales* order. *Allobaculum*, a Gram-positive non-spore-forming anaerobic rod [90], was previously shown to be one of the intestinal genera that are the most sensitive to change in host diet [87]. *Allobaculum* was also found to be enriched after exercise [91] and increased by supplementation with algal dietary fibers [92] or resistant starch from high-amylose maize, and its abundance was positively correlated with the proportions of *Bifidobacterium* and negatively with *Turicibacter* [93]. We did observe a strong inverse relationship between the genera *Allobaculum* and *Turicibacter* (rho=-0.60996, $P<0.0001$), as well as the classes *Erysipelotrichi* and *Clostridia*, suggesting these are competing bacteria in the gut. Additionally, the major end product of *Allobaculum* fermentation is butyrate, which is of particular relevance in the gut because this short chain fatty acid is rapidly taken up by colonocytes, where it serves as a main energy source [94] and has been shown to stimulate the expression of gut peptides (PYY and proglucagon) in cecal tissues [95]. It was proposed that butyrate generation by gut microbes has generally been associated with beneficial effects, including satiety promotion, rather than with obesogenic features, indicating that more complex mechanisms related to fatty acid metabolism could link *Firmicutes* with obesity

[96]. Cecal proportion of *Allobaculum* was directly related to digestive tract weight and to the quantity of food consumed after an overnight fast [93]. Furthermore, the abundance of *Allobaculum* was strongly inversely correlated with the amounts of circulating leptin and expression of several genes correlated with energy expenditure homeostasis and inflammation [87]. It was suggested that *Allobaculum* and several other bacterial are associated with energy homeostasis in the body and the prevention of detrimental age-associated declines in food consumption [93]. Contrary to the finding that *Allobaculum* produces butyrate to be absorbed by the intestinal coloncytes, which would reduce the total energy content in feces, we found that *Allobaculum* was negatively associated with digestive efficiency, thus positively associated with fecal energy density. This suggests that the butyrate produced by *Allobaculum* is unlikely to be entirely absorbed by the host, and that *Allobaculum* might affect energy absorption/extraction from intestinal food. We also observed a weak but significant positive correlation between the abundance of *Allobaculum* and food intake (rho=0.15452, P=0.0338) and a negative correlation with body weight (rho=-0.21615, P=0.0028).

The *Clostridia* class and the *Clostridiales* order are the dominant bacteria among *Firmicutes*, and they have been shown to be influenced by high-fat feeding or obesity. Ingestion of a high-fat diet was associated with an increase in *Clostridiales* compared with a low-fat diet, regardless of propensity for obesity [97]. It was also found that the proportion of *Clostridia* was significantly lower in diabetic patients than in controls and showed a tendency to decrease with higher levels of plasma glucose [98]. This evidence suggests that the *Clostridia* bacterial lineage might be associated with fat digestion and glucose homeostasis. Here, we found the *Clostridia* class and the *Clostridiales* order to

be positively associated with digestive efficiency (and negatively correlated with fecal energy contents), but in weak correlation with food intake (rho=-0.14239, P=0.0506) and body weight (rho=0.17365, P=0.0169). Two bacterial OTUs under the *Clostridia* class found to be significantly correlated with digestive efficiency were: the *Anaerovorax* genus and *Ruminococcus.gnavus*. Both of these two bacteria lineages have fermentative metabolism features, but the *Anaerovorax* genus often metabolizes amino acids [99], while *Ruminococcus* uses carbohydrates as substrate [100]. The major products are short-chain fatty acid such as acetate and butyrate. Additionally, *Ruminococcus.gnavus* was found to be enriched in obese rats [91]. The significant negative correlation with fecal energy content in our study confirms the literature that *Anaerovorax* and *Ruminococcus* helped with extraction of energy from intestinal food.

Other bacteria lineages that are significantly correlated with digestive efficiency include the *Turicibacter* genus. *Turicibacter* is an anaerobic, Gram-positive, rod-shaped bacterium among the *Bacilli* class identified in 2002 and related to fermentative metabolism with maltose and 5-ketogluconates as the only carbohydrate substrates [101]. *Turicibacter spp*. have been detected in the gastrointestinal tracts of several mammals, including humans and mice, as part of the core measurable microbiota [102]. *Turicibacter* was greatly increased after consumption of fermented dairy products in mice [103], which again suggests it has fermentative features. Abundance of the genera *Turicibacter* was negatively correlated with food intake after fasting and gut weight, as well as the PYY expression level [93]. Here in this study, we found that the abundance of the *Turicibacter* genus was relatively low (3.93%±0.27%), and the abundance of *Turicibacter* was positively correlated with body weight (rho=0.42692, P<0.0001), but

not with food intake (rho=0.06976, P=0.3402). The discrepancy in the correlation of *Turicibacter* and food intake between the literature and our study could partially be explained by the fact that the food intake was manually manipulated in our study, and the animals were not under fasting but fixed calorie restriction.

Including food intake and body weight as covariates in the statistical model only resulted in the *Allobaculum* lineage, the *Clostridia* class and the *Anaerovorax* genus having statistical significance. This suggests the variables of food intake and body weight could explain the disappearance of other OTUs. Specifically, the *Turicibacter* genus and unidentified OTUs under the *Bacilli* class were no longer significantly correlated with digestive efficiency after controlling for body weight but were significantly correlated with body weight. This was tested further with body weight and food intake as the only dependent variables, and these two bacteria lineages remained significant (see Table 4). However, none of these significant OTUs in this model was significant in the results of 1a, which tested the correlation between body weight and OTUs in separate samples at different time points. This evidence indicates food intake and body weight could be confounding factors in the analyses of microbiome.

Inclusion of the samples at time point 5 (week 88, second body weight trough) only or 8 (week 103, second body weight peak) generated only different significant OTUs, which suggested time points (or aging) could influence digestive efficiency or OTU abundance. When these two time points were combined, only the *Allobaculum* and *Clostridia* lineages were significantly associated with digestive efficiency. Within the WC group only, again the *Allobaculum* and *Clostridia* lineages showed significance in the association with digestive efficiency. This proves that these two bacterial lineages

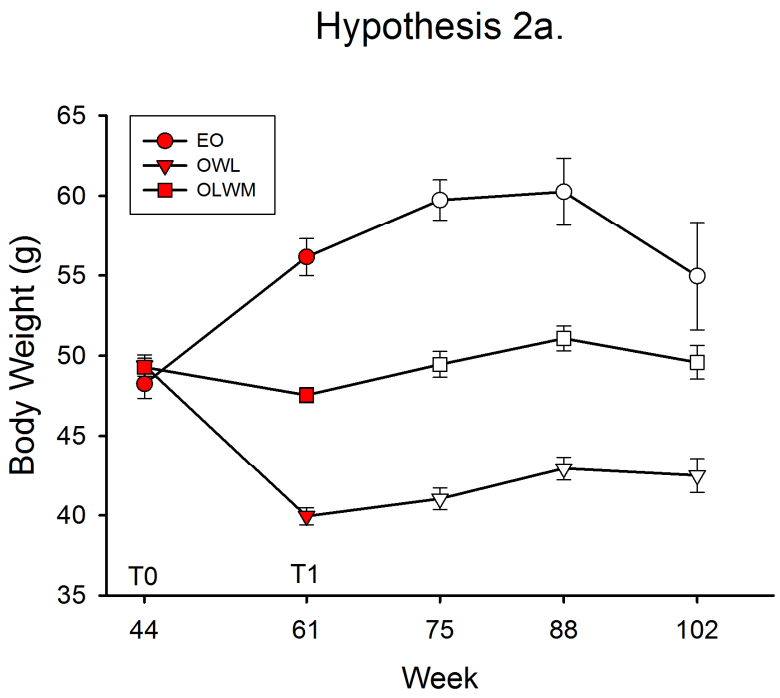
with high abundance are associated with digestive efficiency (independent of dietary treatment and body weight). Although within the WC group, *Allobaculum* was significantly associated with food intake, the correlation coefficient was low.

Overall, the bacteria that are related with digestive efficiency are primarily *Firmicutes* and have fermentation features. The abundances of these bacteria are positively associated with the energy content in feces and consequently negatively associated with the amount of energy absorbed by the host.

Hypothesis 2a.

Fecal Bacteria Compositions (or Specific Strains of Bacteria) Would Have Similar Changes in Mice under Different Levels of Caloric Restriction Compared with *Ad Libitum*–Fed Mice

Figure 12. Average body weight for samples in hypothesis 2a (highlighted in red).



Results

This hypothesis tested the effects of calorie restriction on gut microbiome composition changes. Specifically, there were two levels of gradual calorie restriction over 17 weeks (from 44 weeks to 61 weeks, from time points 0 to 1): OWLM for approximately 15% of restriction and OWL for about 25%, in addition to the *ad libitum*–fed EO group. Within each group, there were no significant differences in the Chao1 index between time points 0 and 1 ($P=0.2083$), but significant differences existed in the Shannon index between these two time points ($P=0.0340$) (**Figure 13**). Within each time point, there were significant differences between any two of the three groups in both the Chao1 and Shannon indices (all $P<0.001$). The PCoA plots (**Figure 14**) revealed distinct clustering or separations between two time points for the Bray-Curtis, unweighted UniFrac and weighted UniFrac. The groups were distinctly separated from each other in the unweighted UniFrac plots.

Figure 13. Alpha diversity for hypothesis 2a (by group for A and B; by time point for C and E; T0: pre-randomization/week44; T1: first body weight trough/week 61; different letters represent significant differences among groups).

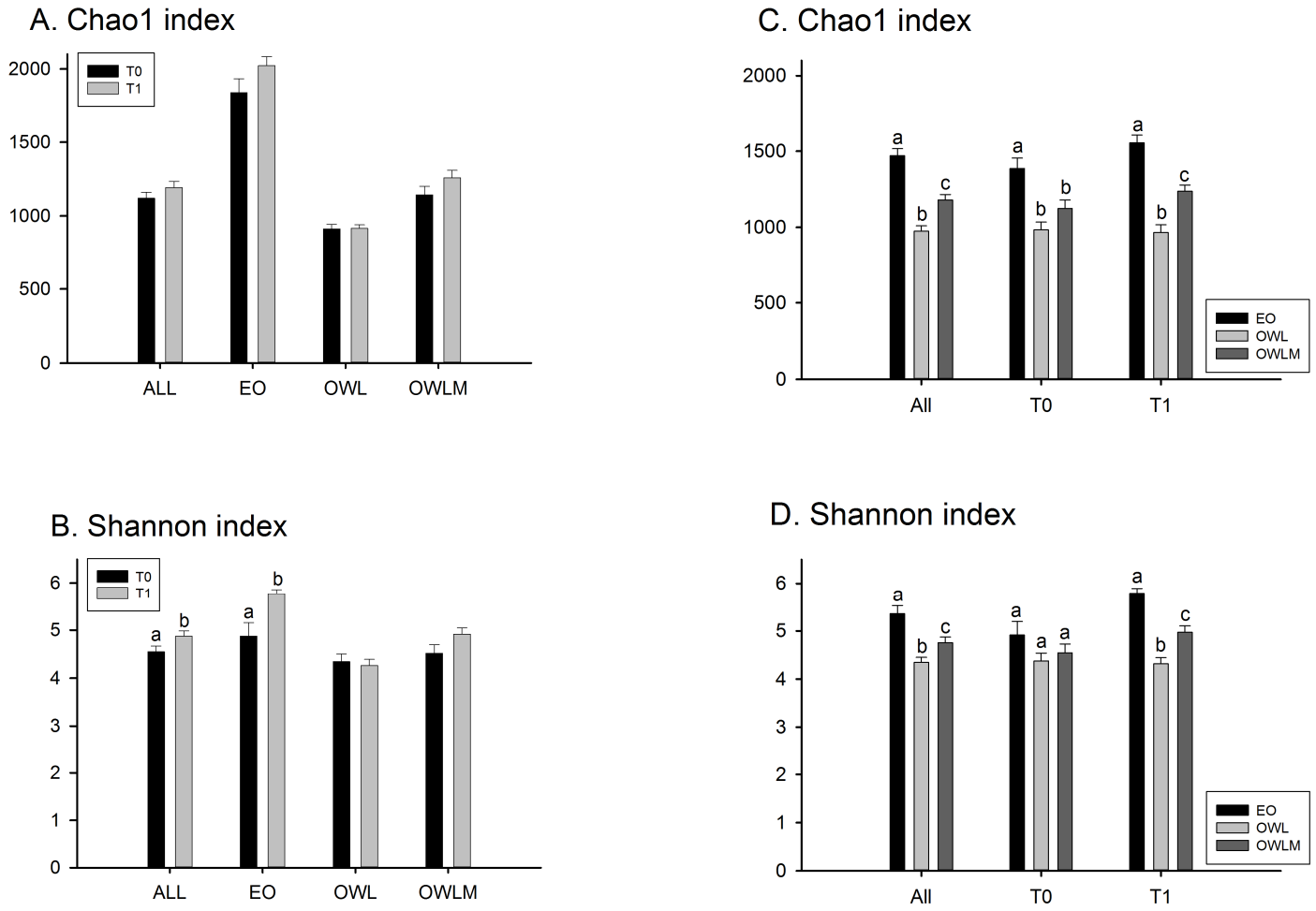
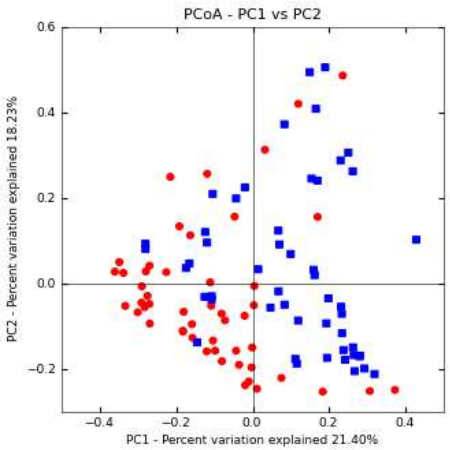
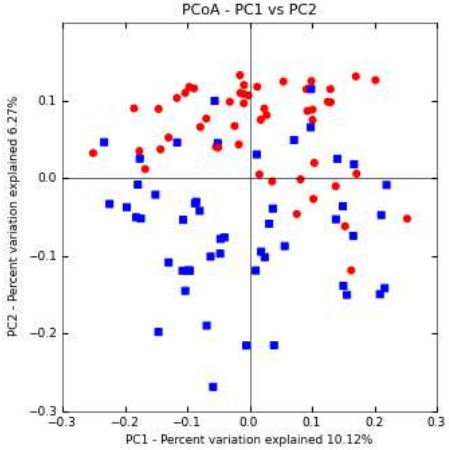


Figure 14. Beta diversity for hypothesis 2a. For A–C by time point: red – T0, blue – T1; for E–G by group: red – EO, green – OWL, blue – OWLM.

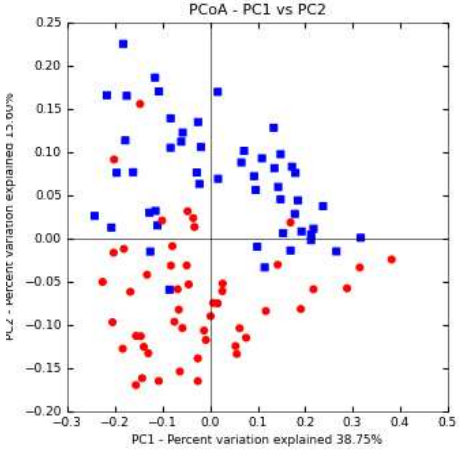
A. Bray-Curtis for time points



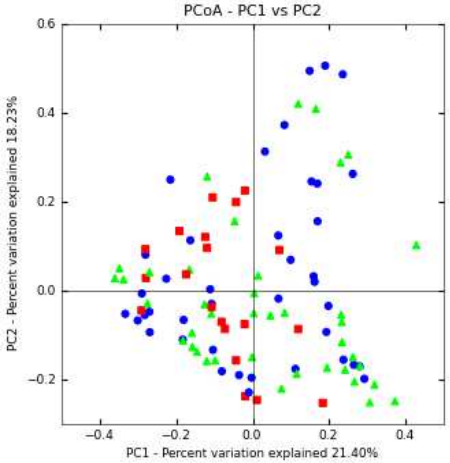
B. unweighted UniFrac for time points



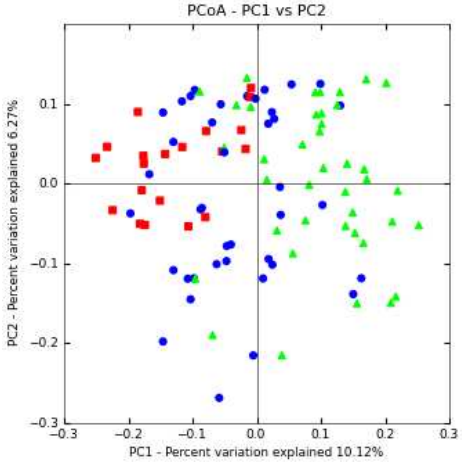
C. weighted UniFrac for time points



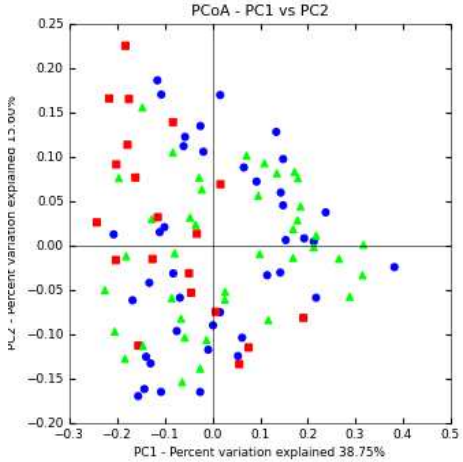
E. Bray-Curtis for groups



F. unweighted UniFrac for groups



G. weighted UniFrac for groups



Regression models were applied to test the specific OTU differences between time points controlling for body weight, food intake and group with animal number as random effect: %OTU = time point + BW +FI + group + animal ID. The results were shown in **Table 9**. Several bacterial lineages were significantly different between time points 0 and 1: the *Clostridiaceae* family, the *Turicibacter* genus, the *Adlercreutzia* genus, the *Clostridium* genus and the *Ruminococcus* genus. Of these, *Clostridiaceae* (18.60%±1.45%)) and *Turicibacter* (10.51%±1.00%) had relatively high abundance. Some bacterial lineages were not significantly different between time points but were significantly associated with body weight, food intake and group assignment. For example, the high-abundance *Allobaculum* genus was significantly associated with body weight. To distinguish the changing directions of these significant bacteria, the adjusted means and standard errors of representative bacteria were used to make figures by group and time point, as shown in **Figure 15.** From the figure, we can see that for the *Turicibacteraceae* family (**Figure 15.A**) and the *Clostridiaceae* family (**Figure 15.B**) (under the *Bacilli* class, **Figure 15.E**), EO remained relatively unchanged from time points 0 to 1; however, the other two restricted groups had a great decrease in abundance. For the unidentified species under the *Clostridiales* order (**Figure 15.C**), the changing directions of this bacterium species were similar to the changing directions of body weight for these three groups. *Allobaculum* was not significantly different between the two time points in this model. However, *Allobaculum* (**Figure 15.D**) had the exact opposite changing directions from their body weight: while EO had a body weight increase from time points 0 to 1, and its *Allobaculum* abundance decreased; OWLM had moderate body weight reduction through ~15% food restriction, but its *Allobaculum*

abundance increased moderately; OWL had more reduction in body weight than OWLM, but its *Allobaculum* abundant increased more than OWLM. The abundance of *Adlercreutzia* increased from time point 0 (0.07%±0.01%) to time point 1 (0.14%±0.01%). Due to its low abundance, the changes of its abundance were not related to the changes in body weight.

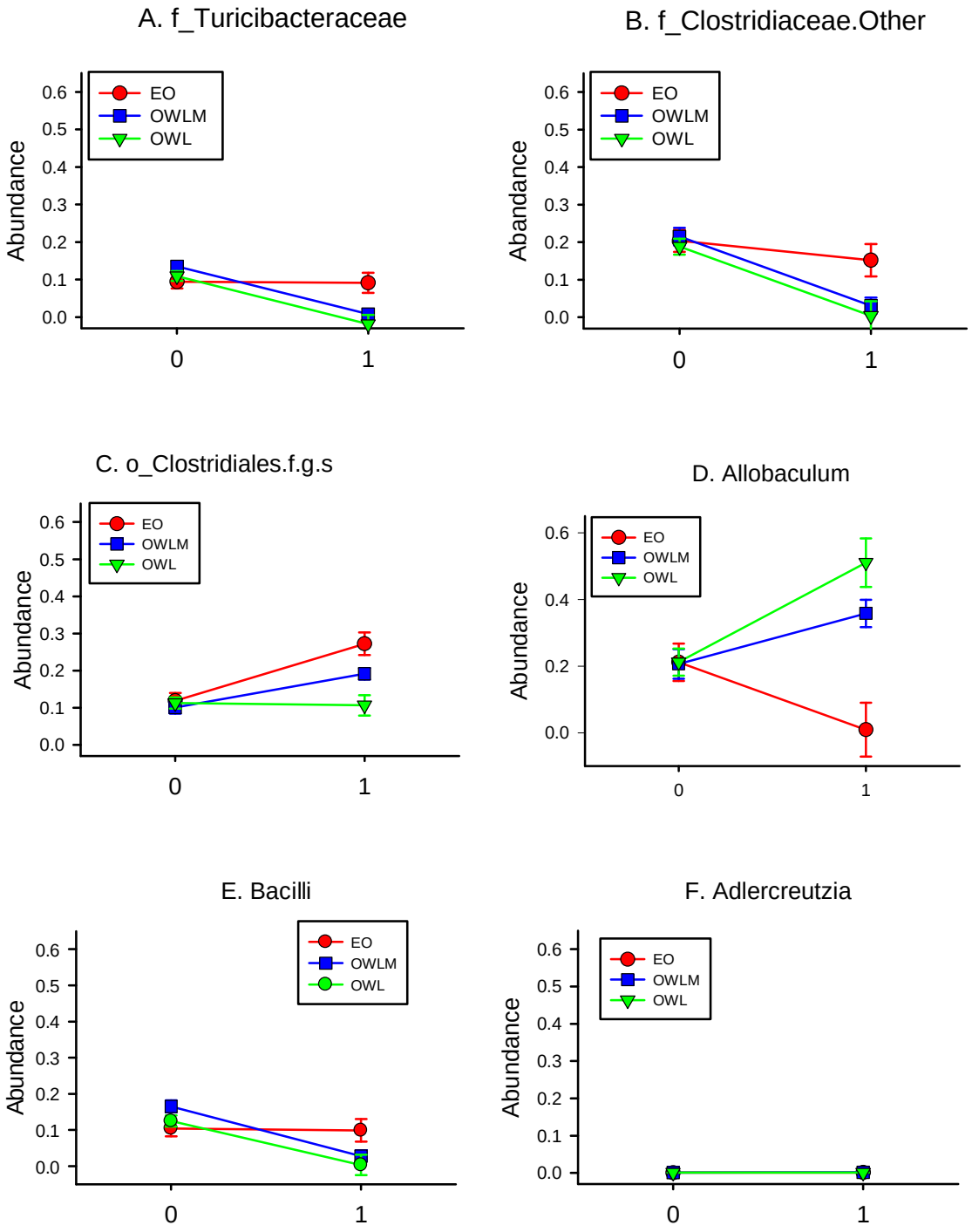
Table 9. Significant OTUs for comparing time points for hypothesis 2a (P-value cutoff: 0.0003).

	mean T0	s.e.	mean T1	s.e.	Time point	Body weight	Time point	Group
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.Other	18.60%	1.45%	6.01%	1.06%	**8.80E-09**	0.00131	8.80E-09	0.587753
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.Other.Other	18.60%	1.45%	6.01%	1.06%	**8.80E-09**	0.00131	8.80E-09	0.587753
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae	19.37%	1.49%	6.54%	1.11%	**1.14E-08**	0.001084	1.14E-08	0.718905
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_Clostridium.s_celatum	0.04%	0.00%	0.01%	0.00%	**5.34E-07**	0.00346	5.34E-07	0.005118
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales	10.51%	1.00%	2.54%	0.51%	**5.86E-07**	0.001175	5.86E-07	0.285368
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae	10.51%	1.00%	2.54%	0.51%	**5.86E-07**	0.001175	5.86E-07	0.285368
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter	10.51%	1.00%	2.54%	0.51%	**5.86E-07**	0.001175	5.86E-07	0.285368
k_Bacteria.p_Firmicutes.c_Bacilli.o_Turicibacterales.f_Turicibacteraceae.g_Turicibacter.s_	10.51%	1.00%	2.54%	0.51%	**5.86E-07**	0.001175	5.86E-07	0.285368
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia	0.07%	0.01%	0.14%	0.01%	**9.21E-07**	0.003982	9.21E-07	0.414405
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia.s_	0.07%	0.01%	0.14%	0.01%	**9.21E-07**	0.003982	9.21E-07	0.414405
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_	10.19%	0.58%	18.06%	1.31%	**1.23E-06**	0.039259	1.23E-06	0.002831
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.g_	10.19%	0.58%	18.06%	1.31%	**1.23E-06**	0.039259	1.23E-06	0.002831
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.g_.s_	10.19%	0.58%	18.06%	1.31%	**1.23E-06**	0.039259	1.23E-06	0.002831
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_Clostridium	0.34%	0.04%	0.16%	0.02%	**2.77E-06**	0.038943	2.77E-06	0.02194
k_Bacteria.p_Firmicutes.c_Bacilli	12.86%	1.15%	4.06%	0.65%	**4.53E-06**	0.000976	4.53E-06	0.135871
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_Clostridium.Other	0.27%	0.03%	0.11%	0.02%	**1.19E-05**	0.037447	1.19E-05	0.011337
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus.s_	0.87%	0.07%	1.36%	0.11%	**1.62E-05**	0.801357	1.62E-05	**0.000108**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus	0.88%	0.07%	1.36%	0.11%	**1.64E-05**	0.781429	1.64E-05	**0.000117**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium	0.04%	0.00%	0.07%	0.01%	**2.53E-05**	0.659869	2.53E-05	0.009083
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium.s_methylpentosum	0.04%	0.00%	0.07%	0.01%	**2.53E-05**	0.659869	2.53E-05	0.009083
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.Other	2.43%	0.20%	4.18%	0.38%	**8.70E-05**	0.908299	**8.70E-05**	0.041146
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.Other.Other	2.43%	0.20%	4.18%	0.38%	**8.70E-05**	0.908299	**8.70E-05**	0.041146
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.Other.Other.Other	2.43%	0.20%	4.18%	0.38%	**8.70E-05**	0.908299	**8.70E-05**	0.041146
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53	0.11%	0.02%	0.24%	0.03%	**0.000158**	0.003727	0.000158	**0.000181**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53.s_	0.11%	0.02%	0.24%	0.03%	**0.000158**	0.003727	0.000158	**0.000181**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other	0.46%	0.03%	0.66%	0.06%	0.000795	0.001442	0.000795	**0.000265**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other.Other	0.46%	0.03%	0.66%	0.06%	0.000795	0.001442	0.000795	**0.000265**
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.08%	0.01%	0.03%	0.01%	0.003822	**0.000203**	0.003822	0.052747
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.08%	0.01%	0.03%	0.01%	0.003822	**0.000203**	0.003822	0.052747
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.08%	0.01%	0.03%	0.01%	0.003822	**0.000203**	0.003822	0.052747
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.08%	0.01%	0.03%	0.01%	0.003822	**0.000203**	0.003822	0.052747

Table 9. (Continued)

k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_Blautia	0.07%	0.02%	0.02%	0.01%	0.018949	0.017188	0.018949	**2.42E-07**
k_Bacteria.p_Actinobacteria.c_Coriobacteriia	0.69%	0.11%	1.52%	0.22%	0.038851	**0.000267**	0.038851	0.969394
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales	0.69%	0.11%	1.52%	0.22%	0.038851	**0.000267**	0.038851	0.969394
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae	0.69%	0.11%	1.52%	0.22%	0.038851	**0.000267**	0.038851	0.969394
k_Bacteria.p_Firmicutes.c_Erysipelotrichi	24.51%	2.49%	32.55%	2.72%	0.12895	**0.000201**	0.12895	0.407261
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales	24.51%	2.49%	32.55%	2.72%	0.12895	**0.000201**	0.12895	0.407261
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae	24.51%	2.49%	32.55%	2.72%	0.12895	**0.000201**	0.12895	0.407261
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum	24.25%	2.49%	31.66%	2.70%	0.165881	**0.000208**	0.165881	0.45984
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum .s_	24.25%	2.49%	31.66%	2.70%	0.165881	**0.000208**	0.165881	0.45984

Figure 15. Comparison of representative OTUs by groups between time points 0 and 1. (Time point 0 was randomization point/week 44; time point 1 was first body weight trough point/week 64; between these two time points, EO kept *ad libitum* feeding; OWL and OWLM received different levels of calorie restriction followed by weight loss.)



Discussion

To our knowledge, very few studies have looked at the effects of calorie restriction on gut microbiome changes. Of them, only one looked at gut microbiome changes after different levels of weight loss combining both food restriction and exercise in humans [104]. Earlier studies showed that the nutrient load can influence the gut (fecal) bacterial community structure over short time scales [42]. The study found that the highest weight loss participants had higher counts of certain bacteria, but it lacked a control group to compare with. In our study, we looked at the gut microbiome changes at two levels of calorie restriction (~15% and ~25%, respectively), in addition to an *ad libitum*–fed group (EO) as a control. We not only found a number of bacterial lineages that were significantly different before and after restriction, but we also confirmed that the directions of changes were generally the same in the two restricted groups while opposite to the EO group.

For example, the directions of changes in the abundance of the *Turicibacteraceae* family for these three groups were similar to that of their body weight changes. As previously discussed, *Turicibacter* under the *Turicibacteraceae family* is related to fermentative metabolism, with carbohydrates as the only substrates, and its abundance generally increased after consumption of fermented dairy or high-grain products. The only carbohydrates that *Turicibacter* can utilize are maltose and 5-ketogluconates [101]. In our study, from time points 0 to 1, the EO group kept receiving *ad libitum* feeding, while the other two groups were restricted in daily food intake, which resulted in subsequent lower availability of carbohydrate in the intestines. The carbohydrates in the diet of this study contains corn starch, which can be subsequently hydrolyzed into

maltose. Thus, the diet restriction reduced maltose availability to *Turicibacter*, which, as a result, decreased its abundance. As for *Allobaculum*, its abundance changed in the exact opposite direction as the body weight changes for the three groups. A previous study has suggested that both diet composition and body weight were correlated with *Allobaculum* abundance, but diet composition alone cannot account for changes in its abundance, thus some metabolic or phenotypic changes caused by maintenance of lower body weight must also be involved, as indicated by the strong inverse correlation between circulating leptin and *Allobaculum* abundance [93]. Here in our study, the diet composition was the same for these three groups, but the amount of daily food intake was significantly different. From time points 0 to 1, the group (OWL) having greater weight loss and food restriction had a greater increase in *Allobaculum* abundance, while the group without food intake changes had a significant reduction in abundance. We agree with the literature that the body weight status or food intake amount is related to the abundance of *Allobaculum*. However, since *Allobaculum* has a fermentation feature and produces butyrate, greater food intake indicates greater availability of energy sources, which should result in a higher level of *Allobaculum*. The finding of lower *Allobaculum* abundance under higher food intake in our study contradicts this hypothesis. Therefore, more complicated mechanisms related to the interplay between the abundance of *Allobaculum* and body weight (or food intake) must exist.

In summary, with the same diet and strain of mice, for the first time, we showed that calorie restriction results in certain intestinal bacterial changes in relative proportion, and these changes are body weight dependent.

Hypothesis 2b.

Fecal Bacteria Will Not Respond to Chronic Diet Changes

Results

This hypothesis tested the gut microbiome changes in weight cycling at both short intervals and long intervals. The weight loss and regain stages were analyzed separately, with time point 2 (second body weight peak for the WC group) as the starting point for weight loss and time point 5 (second body weight trough for the WC group) as the starting point for the weight regain stage. The other points during weight loss (time points 3, 4 and 5) were compared to time point 2, and the other points during weight regain (6, 7, 8 and 9) were compared to time point 5. Pairwise comparison of the time points at each stage for the WC group was conducted. Other studies have shown that switch of diet types could result in shifts in gut community structure after a single day and be stabilized by seven days [105]. Here, we didn't look at the gut microbial changes in days; instead, we followed the changes as short as one week. Additionally, we didn't change the type of diet but manipulated the amount of daily feeding, which could prevent the influence of diet composition on gut microbiome.

The weight loss stage was analyzed first (time points 2 to 5: 2 – weight peak, 3 – one week after restriction, 4 – four weeks after restriction, 5 – 11 weeks after restriction or body weight trough). There were no significant group differences in the Chao1 index scores (P=0.5120) (see **Figure 16**). The Shannon index was significantly different between groups (P=0.0072). The weighed UniFrac PCoA plots of bacterial family indicated high levels of systematic variation where more than 50% of the variation was explained by PC1; however, no distinct clustering was observed.

Pairwise comparisons between T2 and other time points controlling for body weight with animal number as random effect were used to detect the OTU differences during weight loss stage (%OTU = time point [pairwise] + body weight + animal ID). Only the few OTUs that were significant in the comparisons were listed in **Table 10**. Of them, two bacterial lineages were significantly different between time points 2 and 3: the *Ruminococcus* genus and the *Clostridium.methylpentosum* species. An unidentified bacterial lineage under *Firmicutes* was significantly different between time points 2 and 3. The *Ruminococcus* genus and one unnamed species under it were also significantly different between time points 2 and 4. The abundances of the unnamed species under *Ruminococcus* during this weight loss stage were shown in **Figure 17.A**. The corresponding body weight changes were shown in Figure **17.B**.

For the weight regain stage, five time points were included in the analysis (time point 5 to 9: 5 – weight trough; 6 – one week after food release; 7 – five weeks after food release; 8 – 16 weeks after food release or weight peak; 9 – one week of restriction after weight peak). As shown in **Figure 18**, there were no significant differences in the Chao1 index (P=0.0504) between these five time points, but there was a significant difference in the Shannon index (P=0.0023). The differences were between time point 5 and time point 8 (P=0.0047), as well as time point 5 and time point 9 (P=0.0190). Similarly, at the weight loss stage, there was no distinct clustering in the PCoA plots, but the weighted UniFrac PC1 explained 45.38% of variation.

Pairwise comparisons between time point 5 and the other four time points detected only one bacterial lineage that was significantly different between time points 5 and 8: the *Allobaculum* genus (P=3.60E-06) (under *Erysipelotrichi* class) (see **Table 11**).

The abundances of *Allobaculum* genus during this weight regain stage were shown in Figure **17.C**. The corresponding body weight changes were shown in Figure **17.D**.

Figure 16. Alpha and beta diversity for weight loss stage. (Time point 2 to 5, weeks 72, 76, 79 and 88, respectively; this period was the second weight loss stage for WC group; see Figure 2 for more detail; red – T2, light green – T3, bright blue – T4, dark blue – T5.)

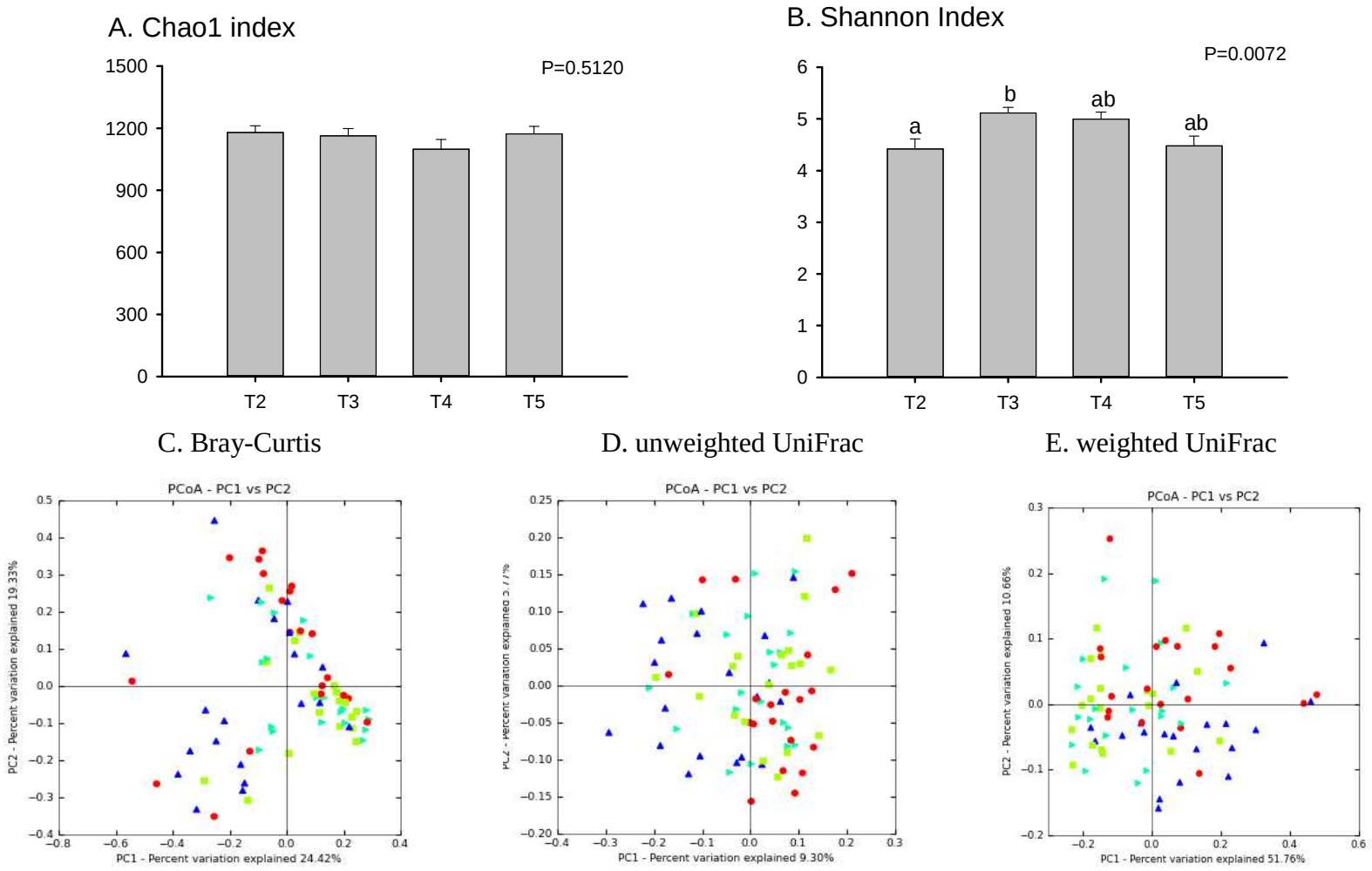


Table 10. Pairwise comparisons in OTU abundances between T2 and other time points during weight loss stage.

OTUs	T2 vs. T3	T2 vs. T4	T2 vs. T5
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus.s_	**3.05E-05**	**2.56E-07**	0.00015242
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium	**3.78E-05**	0.00281167	0.01394163
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium.s_methylpentosum	**3.78E-05**	0.00281167	0.01394163
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus	**6.12E-05**	**5.63E-07**	0.00036868
k_Bacteria.p_Firmicutes.Other	**0.00010215**	**2.48E-06**	0.00031638
k_Bacteria.p_Firmicutes.Other.Other	**0.00010215**	**2.48E-06**	0.00031638
k_Bacteria.p_Firmicutes.Other.Other.Other	**0.00010215**	**2.48E-06**	0.00031638
k_Bacteria.p_Firmicutes.Other.Other.Other.Other	**0.00010215**	**2.48E-06**	0.00031638
k_Bacteria.p_Firmicutes.Other.Other.Other.Other.Other	**0.00010215**	**2.48E-06**	0.00031638
k_Bacteria.p_Firmicutes.c_Clostridia	0.00018843	0.00150961	0.88385154
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales	0.00018843	0.00150961	0.88385154
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae	0.00020228	0.00109424	0.01697571

Table 11. Pairwise comparisons in OTU abundances between T5 and other time points during weight regain stage (only the OTUs in the table between T5 and T8 were significant).

OTUs	T5 vs. T8
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum	3.60E-06
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum.s_	3.60E-06
k_Bacteria.p_Firmicutes.c_Erysipelotrichi	3.74E-06
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales	3.74E-06
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae	3.74E-06

Figure 17. Abundances of *Ruminococcus* and *Allobaculum* during weight loss or weight regain stages T2–T5 in A correspond to the four highlighted red dots in B; T5–T9 correspond to the five red dots in D.

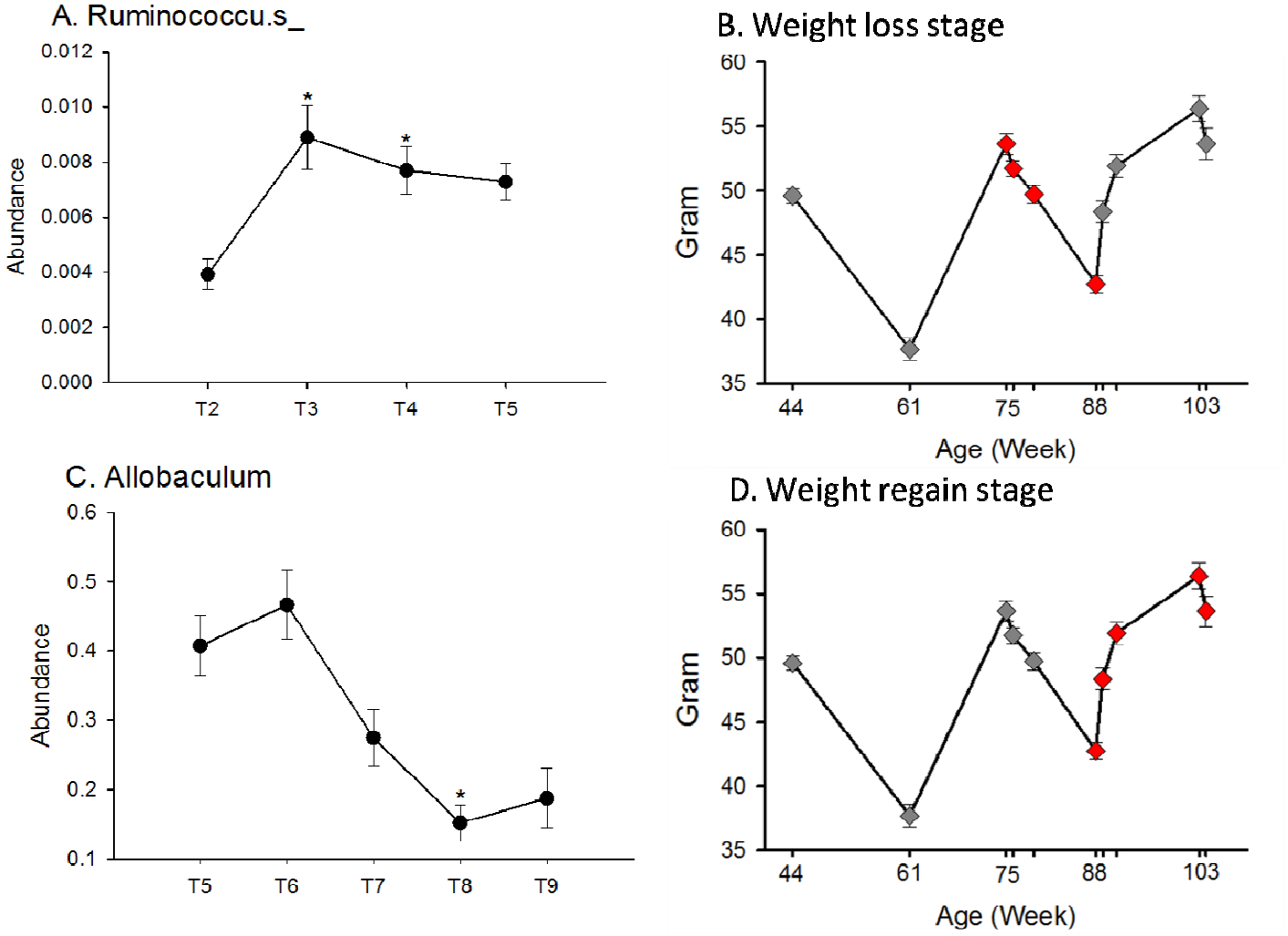
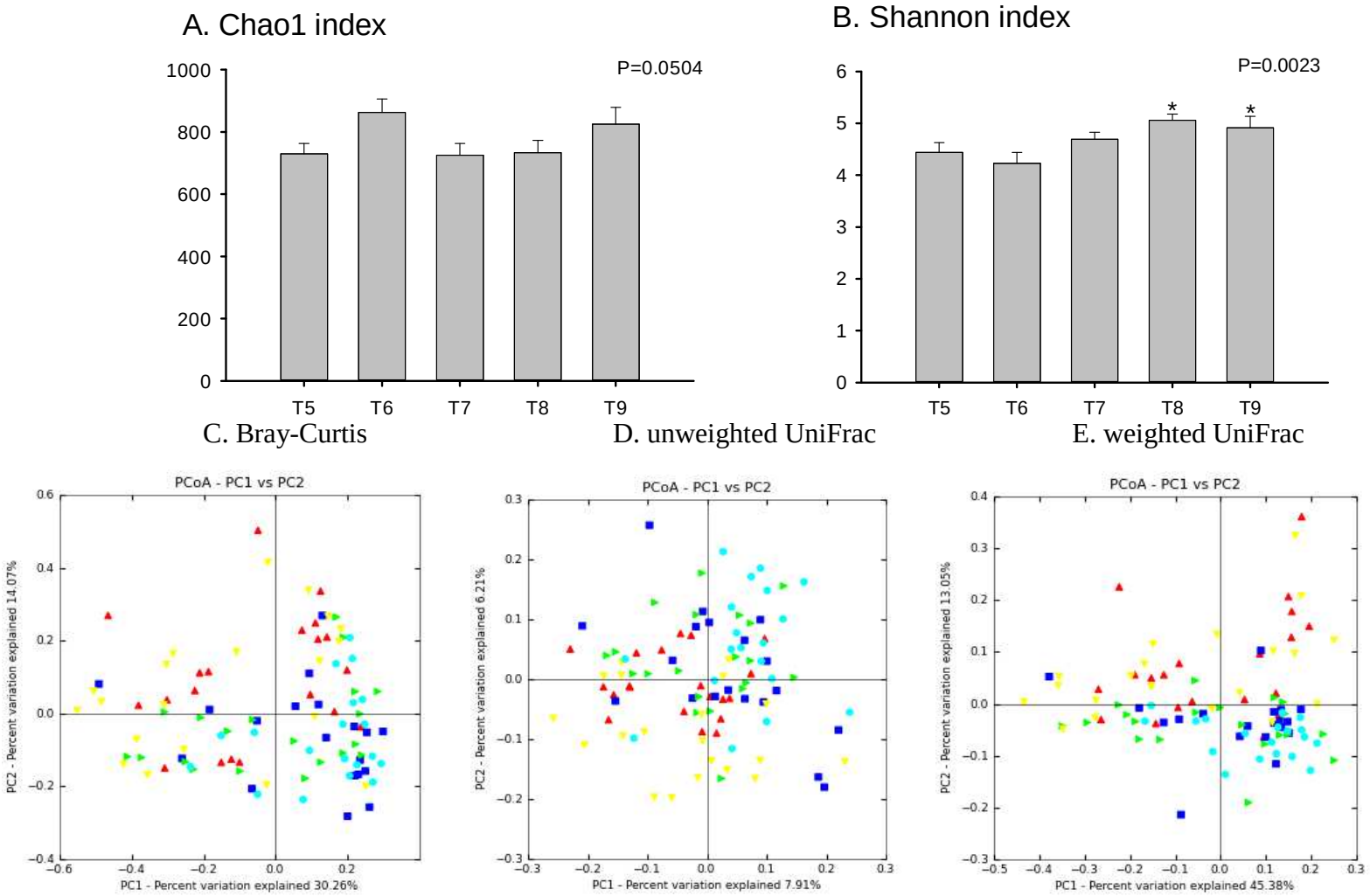


Figure 18. Alpha and beta diversity for weight regain stage. (Time point 5-trough to 8-peak, weeks 88, 89, 81 and 103, respectively; time point 9/week 104 was one week after restriction; this period was the second weight regain stage for the WC group; red – T5, light green – T6, bright blue – T7, yellow – T8; dark blue – T9.)



Discussion

Previous studies have shown that gut microbiome composition could change within days after initiating a new diet, but the enterotype identity remained relatively stable for as long as 10 days [106, 107]. Here in this study, we seek to investigate the long-term gut microbiome changes in mice during a 13-week body weight loss stage induced by gradual calorie restriction, as well as a 15-week weight regain stage caused by *ad libitum* feeding.

During the weight loss stage, the richness of the microbial community (as indicated by the Chao1 index) was not significantly changed, which suggests that the total number of species present in the intestine remains relatively stable during calorie restriction. However, the evenness (as indicated by the Shannon index) first increased but then deceased, which could be associated with the aging process. As for the weight regain stage, since the greatest body weight occurred between time points 5 and 6, which was one week of unlimited food intake after a long term of restriction, the total number of species in the microbial community increased.

In the pairwise comparison of the OTU differences between starting time point and follow-up points of the weight loss stage, one species under the *Ruminococcus* genus was found to be significantly increased from initiation of the food restriction. The genus *Ruminococcus* comprises anaerobic Gram-positive cocci with a fermentative metabolism for which carbohydrates, but no amino acids, serve as substrates for growth and produce acetate, succinate and hydrogen as the major products of glucose metabolism [108, 109]. *Ruminococcus* was also found to be enriched in obese animals [91]. During calorie restriction, with lower availability of carbohydrates to the gut microbial community, the

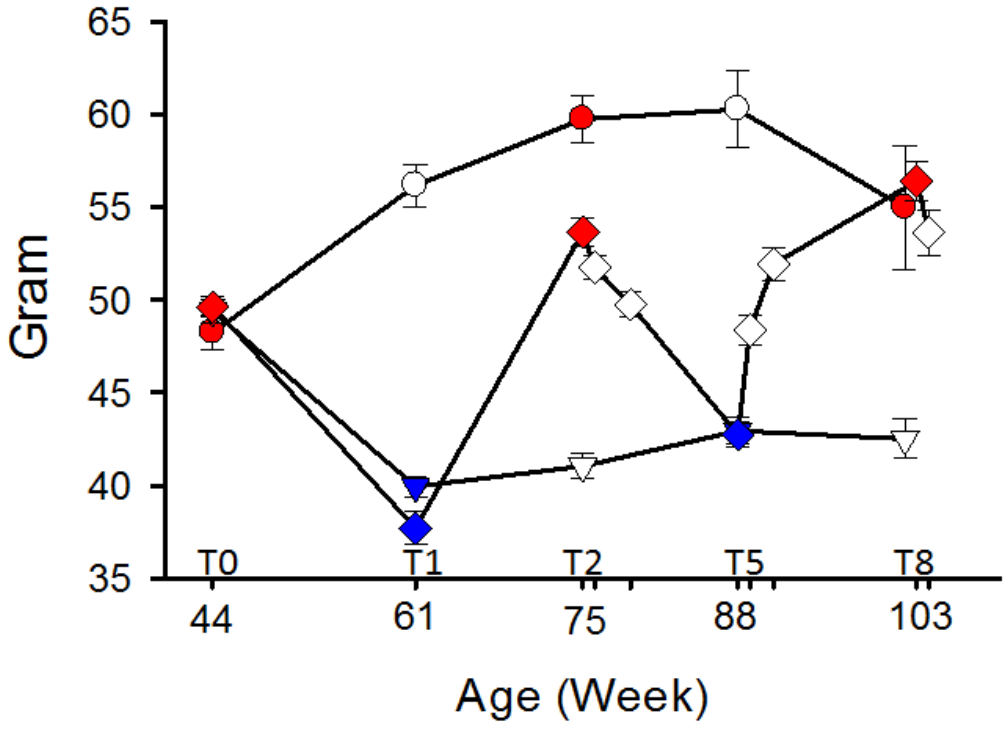
abundance of *Ruminococcus* should decrease. Contrarily, our results show that the abundance of *Ruminococcus* actually increased when there was less substrate supply. Also, when the mice went through calorie restriction, their body weight decreased. This is not in agreement with the literature that obese animals have higher levels of *Ruminococcus.* Overall, this suggests that the abundance of *Ruminococcus* is not simply related to obese status or substrate availability, and there must be more complicated mechanisms associated with the changes of *Ruminococcus.*

During the weight regain stage, only the abundance of *Allobaculum* has significant changes between time points 5 (body weight trough) and 8 (body weight peak). As previously discussed, the abundance of *Allobaculum* changes in the opposite direction as body weight changes. Here its abundance first increased and then decreased, although the constant body weight increased. The possible explanation is that the mice were under food restriction for a long time, which could force them to binge eat when unlimited food was provided suddenly. This is why this week had the greatest body weight increase during the whole weight regain stage. During this week, as a bacterium with fermentative features, the level of *Allobaculum* increased following greater substrate availability. But it is unclear why its abundance began decreasing afterward. Nevertheless, this finding matches the observations in the previous hypothesis.

Hypothesis 2c.

Fecal Bacteria Composition Will Be the Same after the Mice Go through Repeated Weight Loss and Regain Cycles through Calorie Restriction and *Ad Libitum* Refeeding

Figure 19. Average body weight for samples in hypothesis 2c. (Red dots represent body weight peaks of WC and EO at three time points; blue dots represent body weight trough of WC group and OWL at two time points.)



Results

This hypothesis tested the restorability of gut microbiome after repeated weight loss and regain cycles compared to the EO group (as a control for body weight peak) and the long-term restricted OWL group (as a control for body weight trough). Pairwise comparisons were used to compare the OTU abundances between peaks (time points 0, 2 and 8) or between troughs (1 and 5). The model is

as follows: OTU = time point (pairwise) + body weight + animal ID for corresponding groups and time points.

As for the peak-to-peak comparisons, three time points, 0, 2 and 8, were included in the analyses. The alpha diversities were also shown in **Figure 20**. There was no significant difference between these three time points in Chao1 index (P=0.2705). There was a significant difference between time points 2 and 8 in the Shannon index (P=0.0073, overall P=0.0146). In the unweighted UniFrac PCoA plots (**Figure 21.A-C**), the three time points had distinct clustering, but the variation explained by the PC1 was only 7.36%, while in the weighted UniFrac, the three time points were not well separated, but the PC1+PC2 explained 57.66% of the variation. **Figure 22** showed the beta diversity for pairwise comparisons between time points 0 and 8 and between 2 and 8. In all the PCoA plots, the compared two time points were distinctly separated from each other with moderate percentages of systematic variation explained.

The comparison of α diversity between the two body weight troughs of the WC group (time point 1 and time point 5) revealed that the Chao1 index was not significantly different (P=0.4570), but the Shannon index of the first trough was higher than that of the second trough (P=0.0256) (**Figure 20**). These indicated that the second body weight trough had about the same richness of bacteria as the first one but was more evenly distributed than the first trough. β diversity revealed that these two points were clearly separated from each other (**Figure 21**).

Regression models to test the OTU abundance differences were applied as follows: %OTU = time point (pairwise) + body weight + animal ID as random

effect. There was no OTU that reached significance between time points 0 and 2 for the WC group. However, a number of OTUs were significantly different between time points 0 and 8, as well as time points 2 and 9. The results of pairwise comparison between time points 0 and 8 were shown in **Table 12**. From time points 0 to 8, the abundance of *Clostridium.methylpentosum* (under *Ruminococcaceae* family) increased from 0.04%±0.01% to 0.09%±0.01%. The other *Clostridium* genus under Clostridiaceae family decreased from 0.30%±0.03% to 0.09%±0.01%. *Adlercreutzia* genus increased from 0.08%±0.01% to 0.22%±0.02%. *Ruminococcus* genus increased from 0.64%±0.05% to 1.59%±0.18%. *Clostridia* class increased from 57.06%±2.59% to 71.83%±3.03%. *Oscillospira* genus increased from 5.82%±0.42% to 10.54%±0.91%.

The results of pairwise comparison between time points 2 and 8 were shown in **Table 13**. Most of the OTUs that were significantly different between these time points were also significantly different between time point 0 and time point 8, such as *Clostridium.methylpentosum* and *Oscillospira*. However, there were two new OTUs that were not significant in the comparison between time points 0 and 8. The abundance of *Clostridium.ruminantium* decreased from 0.08%±0.01% to 0.02%. Allobaculum decreased from 37.09%±5.15% to 14.30%±2.61%. However, none of the OTUs in any of the pairwise comparisons (between 0, 2 and 8) reached significance for the EO group. Regression models controlling for body weight and animal ID showed that 11 OTUs at various levels were significantly different between time points 1 and 5 (**Table 14**). At class

level, *Bacilli* decreased from 18.7% to 2.3%; *Erysipelotrichi* increased from 20.5% to 41.2%. At order level, *Erysipelotrichales* increased from 20.5% to 41.2%. At family level, *Streptococcaceae* decreased from 1.7% to 0.4%; *Erysipelotrichaceae* increased from 20.5% to 41.2%. These proved that the changes in the *Erysipelotrichi* class were caused by the changes in the *Erysipelotrichaceae* family; however, changes in the *Streptococcaceae* family accounted for only a small portion of the changes in *Bacilli* class. At genus level, *Lactococcus* decreased from 1.7% to 0.4%; *Allobaculum* increased from 20.2% to 40.8% (P=2.25E-05); and *Adlercreutzia* increased from 0.09% to 0.2% (P=0.00023606). These results showed that changes in *Allobaculum* genus were primarily responsible for the changes in the *Erysipelotrichaceae* family, and changes in *Lactococcus* were the primary changes in the *Streptococcaceae* family. Finally, at species level, the two unnamed species under *Allobaculum* and *Adlercreutzia* had the same proportional changes as these two genera, suggesting that changes of these two unnamed species were the causes for changes at the genus level. On the contrary, there was no significant difference in any of the OTUs between time points 1 and 5 for OWL group after Bonferroni correction.

Figure 20. Alpha diversity (A&B. Chao1 and C&D. Shannon Index) for WC group among three body weight peaks. (time points 0, 2 and 8, pre-randomization/week 44; first body weight peak/week 75; second body weight peak/week 102) and between two body weight troughs (time point 1/week 61 and 5/week 88; first and second body weight trough).

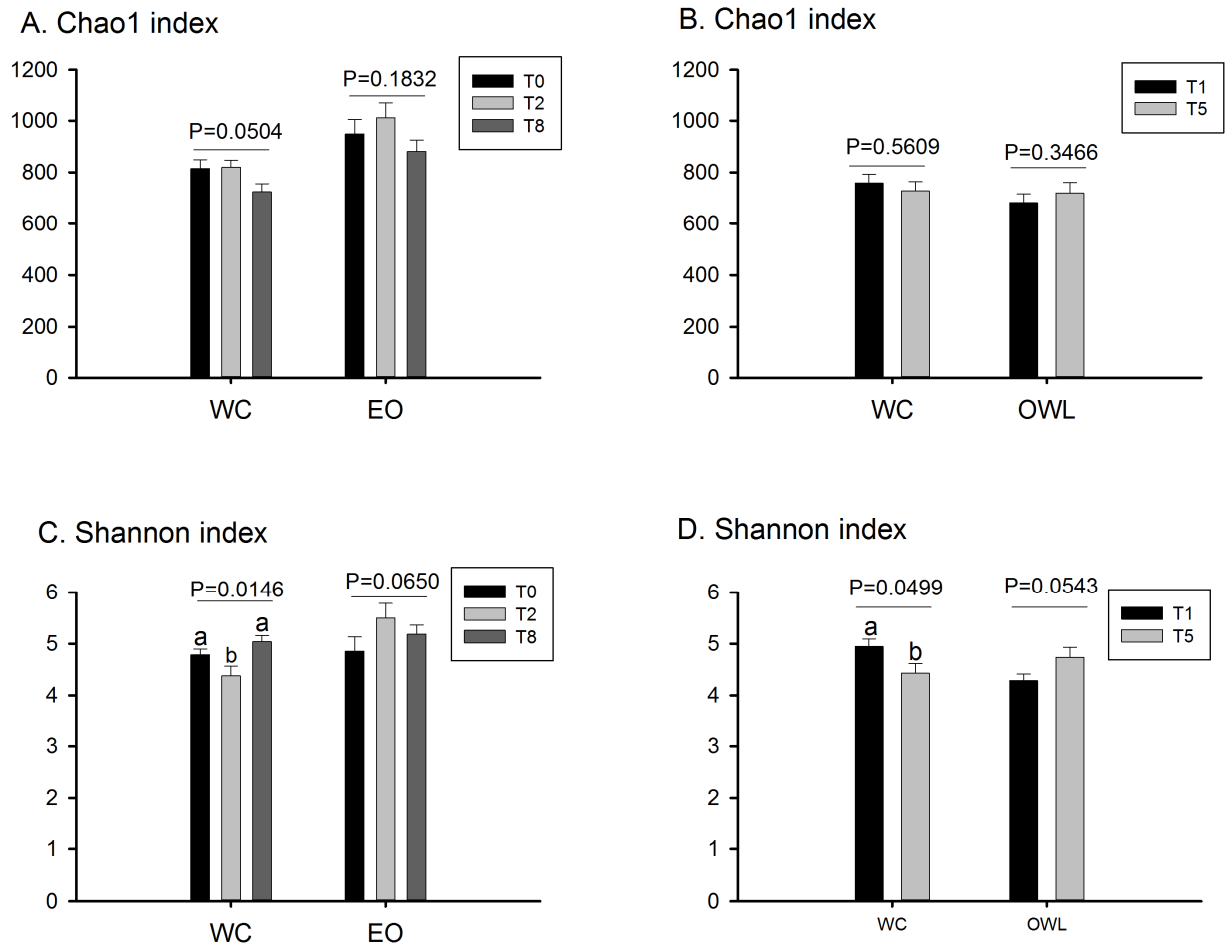
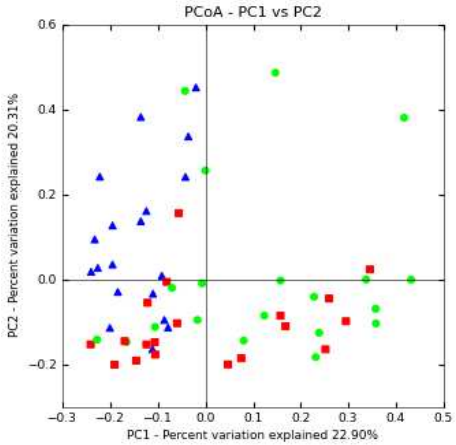
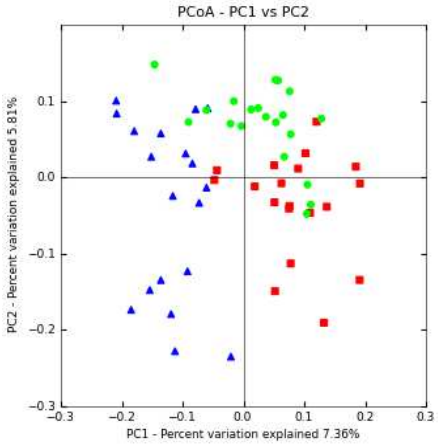


Figure 21. Peak-to-peak comparison in beta diversity. (A. Bray-Curtis; B. unweighted UniFrac; C. weighted UniFrac for three body weight peaks at time points 0, 2 and 8 [weeks 44, 75, 102; red – T0, green – T2, blue – T8]; D. Bray-Curtis; E. unweighted UniFrac; F. weighted UniFrac for two body weight troughs at time points 1 and 5 [weeks 61, 88; red – T1, blue – T5].)

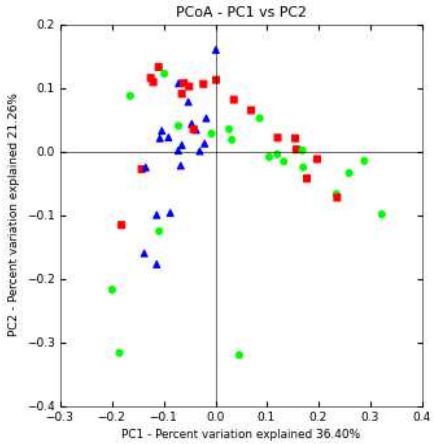
A. Bray-Curtis



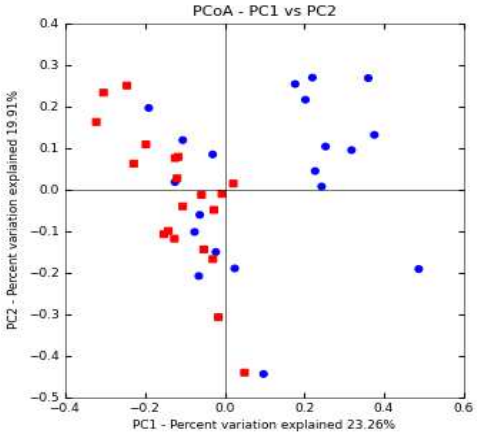
B. unweighted UniFrac



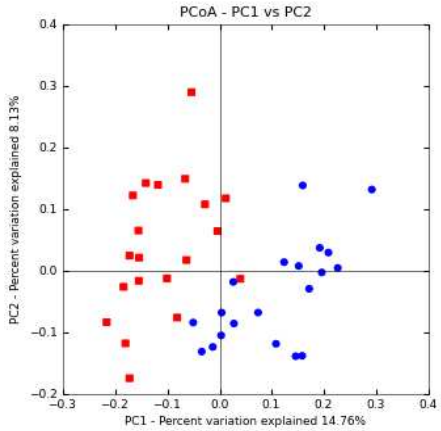
C. weighted UniFrac



D. Bray-Curtis



E. unweighted UniFrac



F. weighted UniFrac

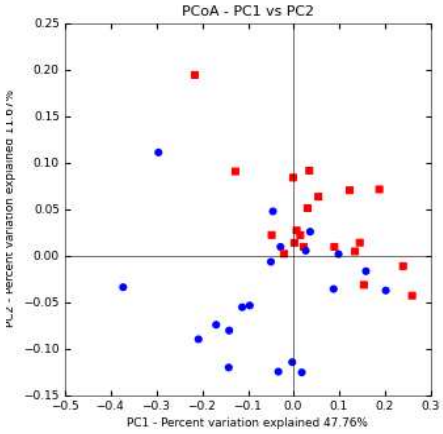
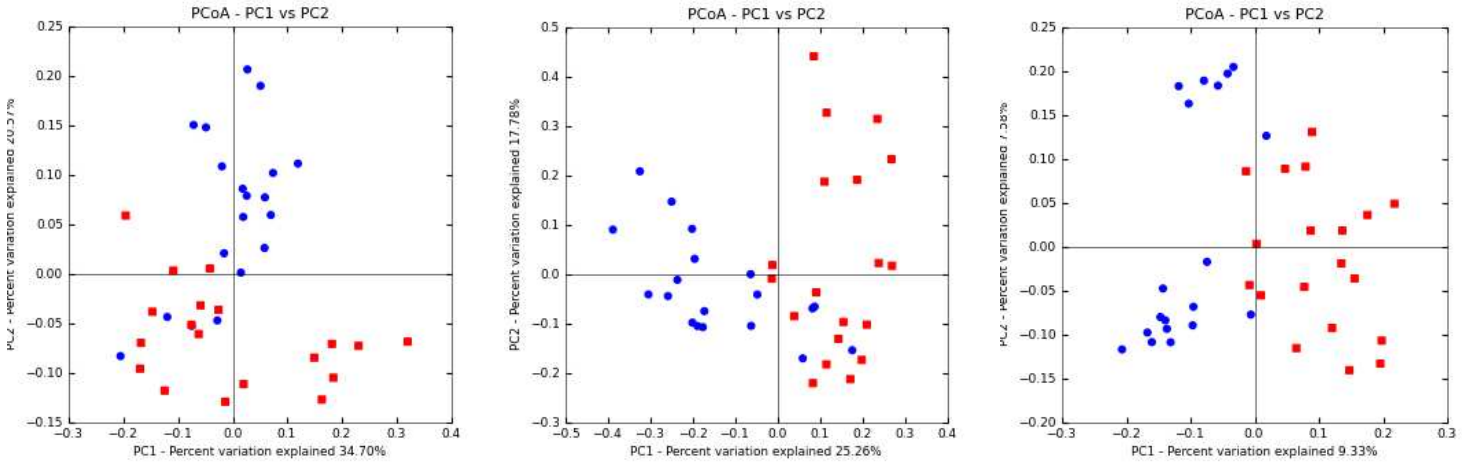


Figure 22. Beta diversity for the comparison between time points 0 (randomization, week 44) and 8 (second body weight regain peak, week 102) and between 2 (first body weight regain peak, week 75) and 8. Red – T0 or T2; blue – T8.

A. Bray-Curtis for T0/T8 B. unweighted UniFrac for T0/T8 C. weighted UniFrac for T0/T8



D. Bray-Curtis for T2/T8 E. unweighted UniFrac for T2/T8 F. weighted UniFrac for T2 and T8

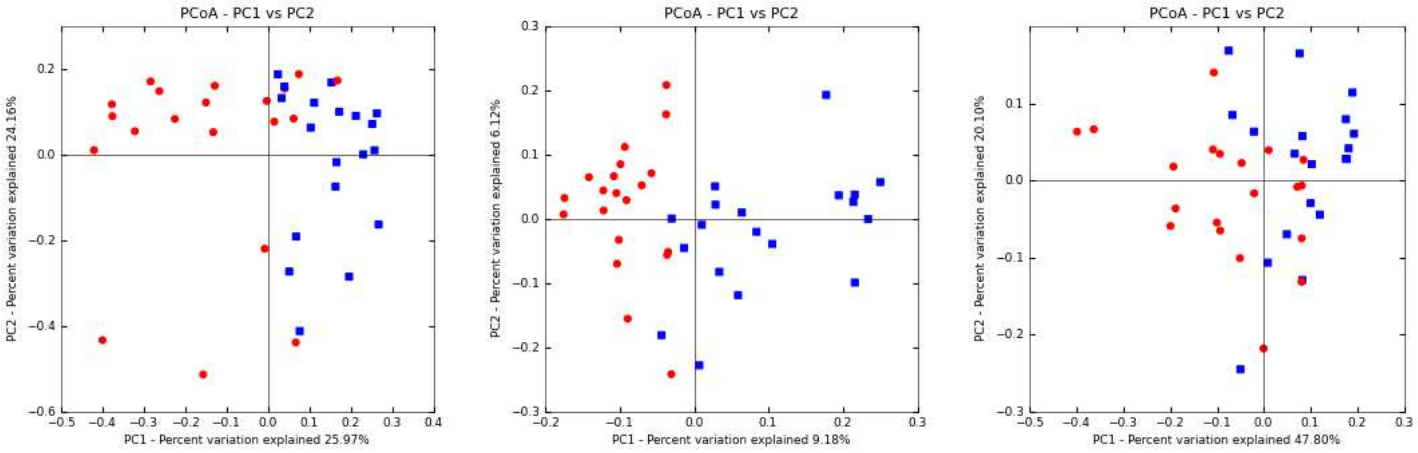


Table 12. OTU comparisons between time points 0 and 8 for WC group (T0 – week 44; T8 – week 102; model controlling for body weight and animal ID, cutoff P-value after Bonferroni correction ≈ 0.0001).

OTUs	mean.T0	s.e.	mean.T8	s.e.	T0 vs. T8	Beta	BW	Beta
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium	0.04%	0.01%	0.09%	0.01%	**3.55E-06**	4.88E-05	0.19298	1.86E-05
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium.s_methylpentosum	0.04%	0.01%	0.09%	0.01%	**3.55E-06**	4.88E-05	0.19298	1.86E-05
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_Clostridium	0.30%	0.03%	0.09%	0.01%	**5.38E-06**	-0.0003	0.8633	7.62E-06
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia	0.08%	0.01%	0.22%	0.02%	**1.23E-05**	0.00018	0.98083	-7.51E-07
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia.s_	0.08%	0.01%	0.22%	0.02%	**1.23E-05**	0.00018	0.98083	-7.51E-07
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_Clostridium.Other	0.23%	0.03%	0.06%	0.01%	**1.41E-05**	-0.0002	0.80989	9.23E-06
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae	9.86%	0.70%	16.31%	1.09%	**2.72E-05**	0.00759	0.76473	0.000547
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus.s_	0.64%	0.05%	1.59%	0.18%	**5.14E-05**	0.00122	0.89348	-3.48E-05
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus	0.64%	0.05%	1.59%	0.18%	**5.22E-05**	0.00122	0.89257	-3.51E-05
k_Bacteria.p_Firmicutes.c_Clostridia	57.06%	2.59%	71.83%	3.03%	**6.84E-05**	0.0151	0.44609	0.004001
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales	57.06%	2.59%	71.83%	3.03%	**6.84E-05**	0.0151	0.44609	0.004001
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_	12.24%	0.75%	22.05%	1.82%	**0.0001**	0.01418	0.47334	-0.001987
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.g_	12.24%	0.75%	22.05%	1.82%	**0.0001**	0.01418	0.47334	-0.001987
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.g_.s_	12.24%	0.75%	22.05%	1.82%	**0.0001**	0.01418	0.47334	-0.001987
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Oscillospira.s_	5.81%	0.42%	10.54%	0.97%	**0.00013**	0.00564	0.82964	0.000321
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Oscillospira	5.82%	0.42%	10.54%	0.97%	**0.00013**	0.00564	0.83133	0.000318
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_Ruminococcus.	1.01%	0.11%	1.91%	0.24%	**0.00019**	0.00097	0.68011	0.000141

Table 13. OTU comparisons between time points 2 and 8 for WC group (T2 – week 61; T8 – week 102; model controlling for body weight and animal ID, cutoff P-value after Bonferroni correction ≈ 0.0001).

Group	mean.T2	s.e.	mean.T8	s.e.	T2 vs. T8	Beta	Body weight	Beta
k_Bacteria.p_Firmicutes.Other	0.15%	0.02%	0.05%	0.01%	**7.63E-06**	-0.00016	0.33467	-2.37E-05
K_Bacteria.p_Firmicutes.Other.Other	0.15%	0.02%	0.05%	0.01%	**7.63E-06**	-0.00016	0.33467	-2.37E-05
K_Bacteria.p_Firmicutes.Other.Other.Other	0.15%	0.02%	0.05%	0.01%	**7.63E-06**	-0.00016	0.33467	-2.37E-05
K_Bacteria.p_Firmicutes.Other.Other.Other.Other	0.15%	0.02%	0.05%	0.01%	**7.63E-06**	-0.00016	0.33467	-2.37E-05
K_Bacteria.p_Firmicutes.Other.Other.Other.Other.Other	0.15%	0.02%	0.05%	0.01%	**7.63E-06**	-0.00016	0.33467	-2.37E-05
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae	9.50%	0.80%	16.31%	1.09%	**9.10E-06**	0.011044	0.83853	0.000381
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium	0.05%	0.01%	0.09%	0.01%	**1.45E-05**	6.97E-05	0.48334	1.03E-05
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Clostridium.s_methylpentosum	0.05%	0.01%	0.09%	0.01%	**1.45E-05**	6.97E-05	0.48334	1.03E-05
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Oscillospira	5.42%	0.47%	10.54%	0.97%	**7.38E-05**	0.008355	0.82256	0.00032
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium	0.08%	0.01%	0.02%	0.00%	**3.52E-05**	-9.94E-05	0.80064	3.35E-06
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium.s_ruminantium	0.08%	0.01%	0.02%	0.00%	**3.52E-05**	-9.94E-05	0.80064	3.35E-06
K_Bacteria.p_Firmicutes.c_Clostridia	50.38%	4.11%	71.83%	3.03%	**3.66E-05**	0.033515	0.52171	0.004571
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales	50.38%	4.11%	71.83%	3.03%	**3.66E-05**	0.033515	0.52171	0.004571
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Oscillospira.s_	5.41%	0.47%	10.54%	0.97%	**7.16E-05**	0.008365	0.82102	0.000323
K_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Alloba culum	37.09%	5.15%	14.30%	2.61%	**7.34E-05**	-0.03338	0.24182	-0.0095
K_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Alloba culum.s_	37.09%	5.15%	14.30%	2.61%	**7.34E-05**	-0.03338	0.24182	-0.0095
K_Bacteria.p_Firmicutes.c_Erysipelotrichi	37.35%	5.16%	14.72%	2.61%	**8.26E-05**	-0.03289	0.2186	-0.00999
K_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales	37.35%	5.16%	14.72%	2.61%	**8.26E-05**	-0.03289	0.2186	-0.00999
K_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae	37.35%	5.16%	14.72%	2.61%	**8.26E-05**	-0.03289	0.2186	-0.00999
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_Anaerovorax	0.02%	0.00%	0.05%	0.01%	0.00015	-0.00016	0.33467	-2.37E-05
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_Anaerovorax.s_	0.02%	0.00%	0.05%	0.01%	0.00015	-0.00016	0.33467	-2.37E-05
K_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.g_Ruminococcus.s_	0.77%	0.09%	1.59%	0.18%	0.00026	-0.00016	0.33467	-2.37E-05

Table 14. OTU comparisons between time points 1 and 5 for WC group (T1 – week 61; T5 – week 88; model controlling for body weight and animal ID; cutoff P-value after Bonferroni correction = 0.0003).

OTUs	mean. T1	mean.T 5	Time point (P)	Body weight (P)	Time point (β)	Body weight (β)
Bacteria.Firmicutes.Bacilli.Lactobacillales.Streptococcaceae	1.71%	0.39%	**1.34E-07**	0.10037119	-0.0028026	-0.0004001
Bacteria.Firmicutes.Bacilli.Lactobacillales.Streptococcaceae.Lactococcus.s__	1.69%	0.38%	**1.56E-07**	0.08885229	-0.0027343	-0.0004165
Bacteria.Firmicutes.Bacilli.Lactobacillales.Streptococcaceae.Lactococcus	1.69%	0.38%	**1.58E-07**	0.08894799	-0.0027333	-0.0004162
Bacteria.Firmicutes.Erysipelotrichi	20.46%	41.20%	**1.86E-05**	0.09395521	0.03573822	0.01274694
Bacteria.Firmicutes.Erysipelotrichi.Erysipelotrichales	20.46%	41.20%	**1.86E-05**	0.09395521	0.03573822	0.01274694
Bacteria.Firmicutes.Erysipelotrichi.Erysipelotrichales.Erysipelotrichaceae	20.46%	41.20%	**1.86E-05**	0.09395521	0.03573822	0.01274694
Bacteria.Firmicutes.Erysipelotrichi.Erysipelotrichales.Erysipelotrichaceae.Allobaculum	20.25%	40.79%	**2.25E-05**	0.10279591	0.0356558	0.01241584
Bacteria.Firmicutes.Erysipelotrichi.Erysipelotrichales.Erysipelotrichaceae.Allobaculum.s_	20.25%	40.79%	**2.25E-05**	0.10279591	0.0356558	0.01241584
Bacteria.Firmicutes.Bacilli	18.73%	2.34%	**2.31E-05**	0.64870749	-0.0383373	-0.0020863
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.Adlercreutzia	0.09%	0.20%	**0.00023606**	0.71784137	0.00028151	-1.33E-05
kacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.Adlercreutzia.s_	0.09%	0.20%	**0.00023606**	0.71784137	0.00028151	-1.33E-05

Discussion

Repeated weight losses and regains (yo-yo dieting) have become a common pattern for obese individuals because of the difficulty of maintaining a weight loss status [55]. To date, there has been no study looking at the gut microbiome changes before and after weight loss and regain cycles. This study aims to test whether gut microbiome is restorable, with special attention to identify the specific bacteria that might be different before and after the cycles. One unique feature of this project is that the weight cycling was manipulated in the absence of diet cycling, which could potentially preclude the influence of different dietary composition on bacterial communities.

Samples at three body weight peaks (baseline, after the first weight cycle and after the second weight cycle) for the WC group and the contemporary EO group and two body weight troughs (the first and second weight losses) for the WC group and the contemporary OWL group were included in the analyses. For the microbial richness, there were no significant differences in the WC/EO groups at the three peak points or in the WC/OWL groups at the trough points. For microbial evenness, the second weight peak was significantly less evenly distributed than the other two peaks, while no difference was observed for the EO group. The second trough point of the WC group also was significantly less evenly distributed than the first trough, while there was no difference in the OWL group at these two time points. In between group diversities, samples at the three peaks were generally separated from each other for the WC group. So were the samples at the two troughs.

Between the first (time point 0) and second peaks (time point 2), no OTUs were significantly different after Bonferroni correction. However, several OTUs were

significantly different between the first peak and third peak (time point 8), as well as between the second and third peaks. Furthermore, there were also some OTUs that were significantly different between the two body weight troughs (time points 1 and 5). Of them, the genera *Allobaculum* and *Adlercreutzia* were significant in the comparisons both between weight peaks and troughs. Most of these bacteria that were significant between these time points have fermentative features, such as the genera *Ruminococcus*, *Allobaculum* and *Lactococcus*, as previously described. *Anaerovorax* is a strictly anaerobic bacterium of fermentative metabolism, often metabolizing amino acids into acetate and butyrate [99]. There were also a few new bacteria that are related to other metabolic functions: The *Adlercreutzia* genus is involved in the conversion of daidzein to equol [110]. The *Clostridium.methylpentosum* species could metabolize rhamnose released via the enzymatic depolymerization of dietary pectin [111]. *Oscillospira* is a large bacterium often observed in the rumen contents of sheep and cattle as well as in the alimentary tract of other herbivorous animals [112]. Its population was found to be rapidly changed when animals changed their diet types [113]. However, it is hard to associate the functions of these bacteria with the weight changes of hosts based on the current findings.

Since mice at these three body weight peak time points all received *ad libitum* feeding, the differences between them were: 1) mice were at different ages; and 2) mice at the later two points went through weight loss or regain cycle(s). Additionally, mice at the two body weight troughs also had these two differences. Nevertheless, the fact that there was no significant difference in any of the OTUs between these three same time points for the EO group and the fact that there was no significant difference between the

two same time points for the OWL group suggest the differences in the WC groups before and after weight cycles are not due to aging. Therefore, going through the weight loss and cycles must have effects on these bacteria. These effects could originate from the physiological changes in hosts after weight cycling or could come from the complicated interaction of the microbial community themselves through cycles of energy source restrictions and availabilities. However, to date there has been insufficient evidence showing weight cycling has effects on behavior, metabolism and health [58, 114, 115]. A situation resembling this is the repeated use of antibiotics: a human study [116] with repeated broad spectrum antibiotic perturbation demonstrated that the intestinal microbial community changes after antibiotic use. However, these changes varied among subjects and between the two courses within subjects. Furthermore, the gut microbiota composition stabilized by the end of the experiment but was altered from its initial state. Similarly, the microbiota after a single administration of antibiotics had only partially recovered to their pre-treatment compositions in some cases [117]. Taken together, the evidence suggests that there is either complex competition within microbial communities, which leads to permanent changes in some bacteria population, or some physiological changes in the host precludes the restorability of initial microbiome composition. Therefore, if a copy of gut microbiome in healthy status can be stored, then later once the host encounters unusual status such as disease status or weight cycles, the previous healthy gut microbiome can still be restored from the copy.

Hypothesis 3a.

Fecal Bacteria Composition Will Be Stable under a Fixed Diet Regimen

Independent of Time Effect

Results

This hypothesis tested the gut microbiome structure and stability for mice under different dietary treatment: one *ad libitum*–fed group and two caloric-restricted groups. About 200 samples at four time points (T1–week 61, T2–week 75, T5–week 88, T8–week 102; EO–n=10, OWL–n=20, OWLM–n=20) were selected. Please refer to Figure 2 for body weight, fat mass, fat-free mass and food intake status of these three groups at four time points.

First, the alpha diversities were viewed by group or time point for all of these samples (**Figure 23**). As seen in the figure, if the samples were separated by groups, there were significant differences in bacterial richness (Chao1 index) between each pair of the three groups (EO vs. OWL, P<0.0001; EO vs. OWLM, P<0.0001; OWL vs. OWLM, P=0.0004), with EO having the highest number of species and OWL the lowest. For bacterial evenness (Shannon index), there were significant differences between the *ad libitum*–fed EO group and both restricted groups (EO vs. OWL, P<0.0001; EO vs. OWLM, P<0.0001) but no statistical difference between OWL and OWLM (P=0.1823). **Figure 24** shows the beta diversity separated by group or time point. The three groups were separated from each other, especially in the unweighted UniFrac PCoA plot. The lesser separation in the weighted UniFrac suggests that major phylogenetic differences were caused primarily by the OTUs with low abundances. However, the scatters divided by time points were not well separated from each other.

To reveal the OTU differences between different time points, a regression model was used as follows: OTU = time point (in pairs) + body weight + animal ID + group. The results of pairwise comparisons between T0 and T1, T0 and T2 and T0 and T8 were

shown in **Tables 15–17**, respectively. Not many OTUs were significant in these pairwise comparisons between time points. Only one bacterial lineage was significantly different between all three comparisons after controlling for body weight and group: the *Adlercreutzia* genus. The scatter plots of the abundance of the *Adlercreutzia* genus were shown in **Figure 25**. From the figure, we can see that the abundance of the *Adlercreutzia* genus became higher at later time points for all groups, OWLM only and OWL only, but not for EO only. However, the body weight variable did not reach significance for this lineage.

To test whether the effects of real age (as a continuous variable) is similar as arbitrary time points in the statistical model, another model was used as follows: OTU = age (week) + body weight + animal ID + group. The results were shown in **Table 18**. The abundance of six bacterial lineages was significantly associated with age: the RF39 order, *Clostridium.ruminantium*, the SMB53 genus, an unnamed class under *Firmicute*s, the *Christensenellaceae* family and the *Adlercreutzia* genus. However, all of these OTUs had a relatively low abundance (0.07% to 0.18%) with small beta coefficient values.

Furthermore, the alpha diversity was also viewed within groups for different time points (**Figure 26**). For the EO group, there was a significant difference only in the Chao1 index between T1 and T8 (P=0.0051). For the OWL group, only the Shannon index was significantly different between T1 and T8 (P=0.0024). And for the OWLM group, there were significant differences between T1 and T2 (P=0.0021), as well as T1 vs. T5 (P=0.0003) in the Chao1 index, but no statistical significance in the Shannon index (P=0.3893).

Figure 23. Alpha diversity by group or time point for hypothesis 3a. (A&B by group; C&D by time point; T1 – week 61; T2 – week 75; T5 – week 88; T8 – week 102; different letters represent significant differences between groups; for these four time points over 40 weeks, EO received *ad libitum* feeding; OWL and OWLM received different levels of restriction with relatively stable body weight.)

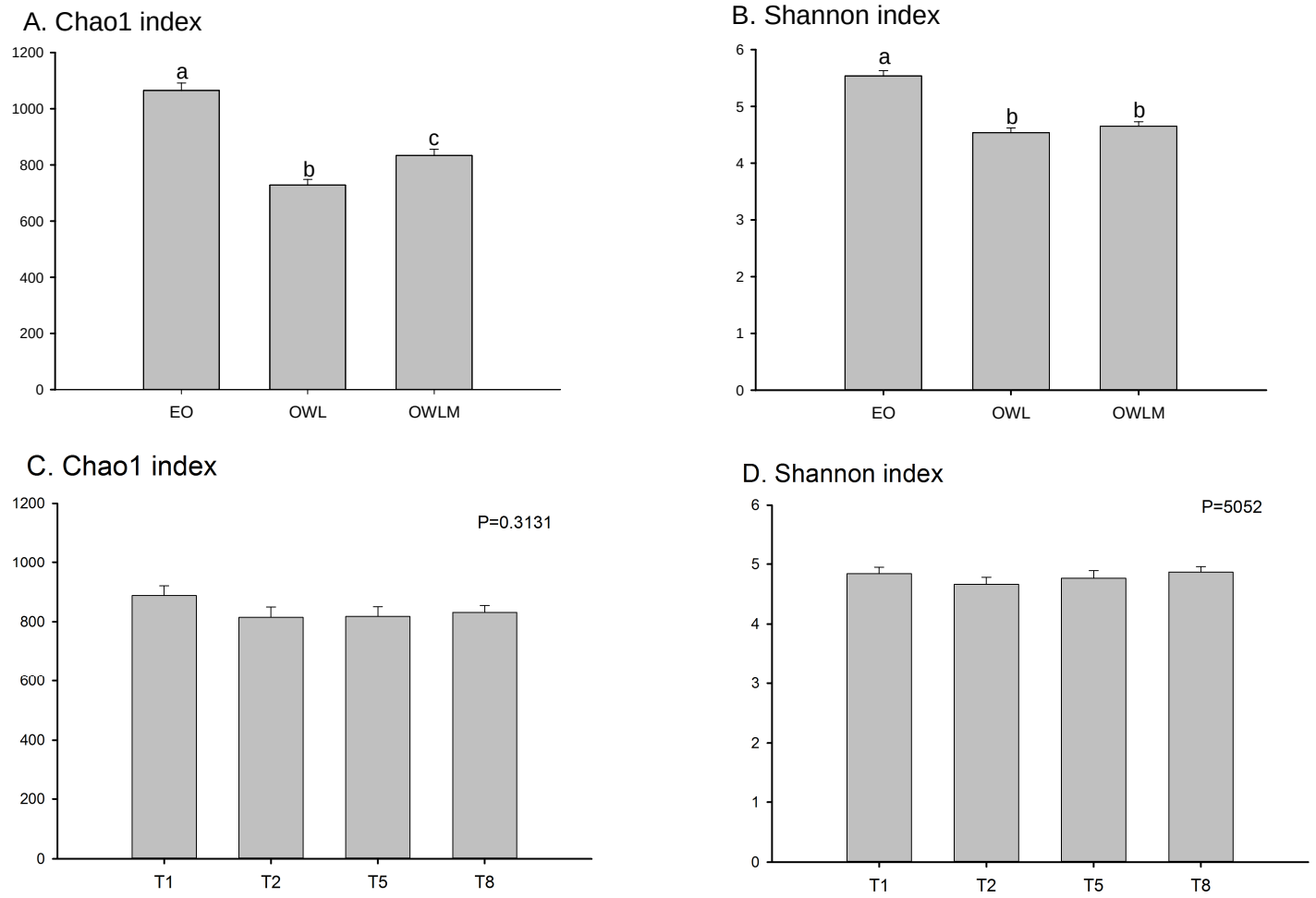
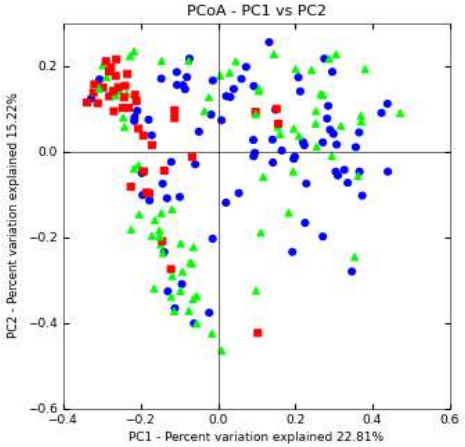
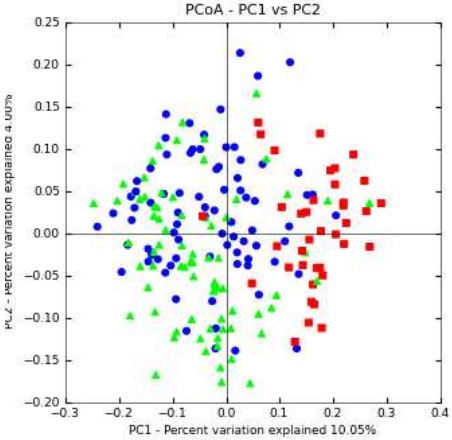


Figure 24. Beta diversity by group (red – EO, green – OWL, blue – OWLM) or time point (red – T1/week 61; green – T2/week 75; bright blue – T3/week 88; dark blue – T4/week102) for hypothesis 3a.

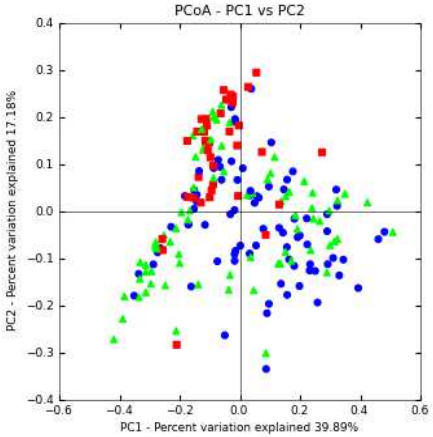
A. Bray-Curtis for three groups



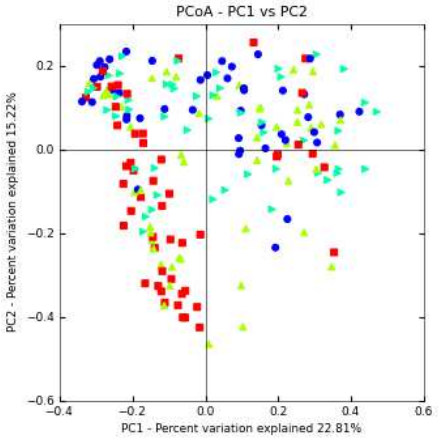
B. unweighted UniFrac for three groups



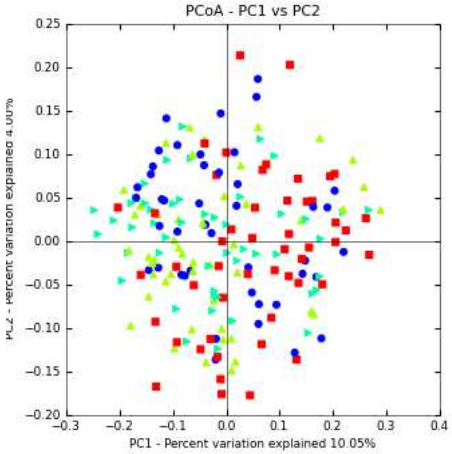
C. weighted UniFrac for three groups



D. Bray-Curtis for four time points



E. unweighted UniFrac for four time points



F. weighted UniFrac for four time points

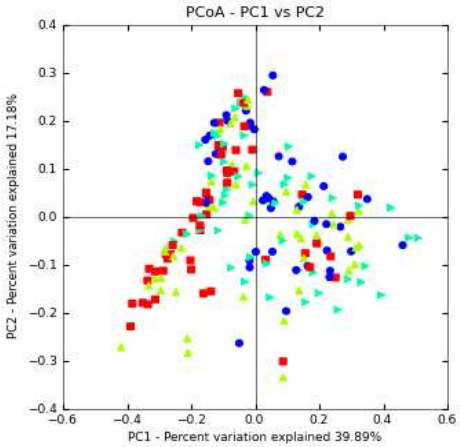


Table 15. Pairwise comparisons (T1 vs. T2) between time points controlling for body weight, animal ID and group (T1 – week 61; T2 – week 75).

OTUs	Abundance	T1 vs. T2	Body weight	Group
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia	0.00185	**1.64E-05**	0.86072	0.03033
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia.s_	0.00185	**1.64E-05**	0.86072	0.03033
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53	0.00139	0.00512	**6.03E-08**	0.02328
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53.s_	0.00139	0.00512	**6.03E-08**	0.02328
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other	0.0055	0.01226	**4.77E-07**	0.00362
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other.Other	0.0055	0.01226	**4.77E-07**	0.00362
k_Bacteria.Other	0.00059	0.01492	**7.35E-07**	0.03111
k_Bacteria.Other.Other	0.00059	0.01492	**7.35E-07**	0.03111
k_Bacteria.Other.Other.Other	0.00059	0.01492	**7.35E-07**	0.03111
k_Bacteria.Other.Other.Other.Other	0.00059	0.01492	**7.35E-07**	0.03111
k_Bacteria.Other.Other.Other.Other.Other	0.00059	0.01492	**7.35E-07**	0.03111
k_Bacteria.Other.Other.Other.Other.Other.Other	0.00059	0.01492	**7.35E-07**	0.03111
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_	0.00128	0.18275	**7.39E-07**	0.04599
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_.s_	0.00128	0.18275	**7.39E-07**	0.04599
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.00037	0.08158	**1.71E-06**	0.00248
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.00037	0.08158	**1.71E-06**	0.00248
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.00037	0.08158	**1.71E-06**	0.00248
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.00037	0.08158	**1.71E-06**	0.00248
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae.g_Lactococcus.s_	0.00681	0.93256	0.00964	**1.05E-07**
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae.g_Lactococcus	0.00681	0.93513	0.00957	**1.06E-07**
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae	0.00705	0.95951	0.00934	**1.82E-07**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae.	0.00158	0.21769	0.26758	**8.62E-07**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_	0.00128	0.16142	0.22148	**3.25E-06**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_.s_	0.00128	0.16142	0.22148	**3.25E-06**

Table 16. Pairwise comparisons (T1 vs. T5) between time points controlling for body weight, animal ID and group (T1 – week 61; T5 – week 88).

OTUs	Abundance	T1 vs. T5	Body weight	Group
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Christensenellaceae	0.00072	**6.84E-10**	0.05815	
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Christensenellaceae.g_	0.00072	**6.84E-10**	0.05815	0.11386
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Christensenellaceae.g_.s_	0.00072	**6.84E-10**	0.05815	0.11386
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia	0.00185	**1.47E-07**	0.00955	0.90988
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia.s_	0.00185	**1.47E-07**	0.00955	0.90988
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53	0.00139	0.00077	**2.30E-05**	0.02971
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53.s_	0.00139	0.00077	**2.30E-05**	0.02971
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other	0.0055	0.01916	**3.31E-05**	0.00409
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other.Other	0.0055	0.01916	**3.31E-05**	0.00409
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae.g_Lactococcus	0.00681	0.29353	0.63592	**2.28E-07**
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae.g_Lactococcus.s_	0.00681	0.29663	0.63484	**2.36E-07**
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae	0.00705	0.2718	0.67465	**3.05E-07**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae	0.00035	0.00399	0.16943	**2.84E-05**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae.g_Dehalobacterium	0.00035	0.00399	0.16943	**2.84E-05**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae.g_Dehalobacterium.s_	0.00035	0.00399	0.16943	**2.84E-05**

Table 17. Pairwise comparisons (T1 vs. T8) between time points controlling for body weight, animal ID and group (T1 – week 61; T8 – week 102; n=10 for EO; n=20 for OWL; n=20 for OWLM).

	Abundance	T1 vs. T8	Body Weight	Group
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia	0.00185	**7.62E-07**	0.01311	0.00162
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia.s_	0.00185	**7.62E-07**	0.01311	0.00162
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.00037	0.01865	**9.15E-07**	0.01471
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.00037	0.01865	**9.15E-07**	0.01471
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.00037	0.01865	**9.15E-07**	0.01471
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.00037	0.01865	**9.15E-07**	0.01471
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_	0.00128	0.07993	**2.68E-05**	0.00959
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_.s_	0.00128	0.07993	**2.68E-05**	0.00959
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae.	0.00158	0.86355	0.90363	**2.47E-06**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_	0.00128	0.54353	0.91692	**1.65E-05**
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_.s_	0.00128	0.54353	0.91692	**1.65E-05**

Table 18. Aging effects on the microbiome changes (T1 – week 61; T8 – week 102; n=10 for EO; n=20 for OWL; n=20 for OWLM; for time points, weeks 61, 75, 88 and 102 as an dependent continuous variable).

	OTU abundance	P.Week	B.Week	P.BW	B. BW	P.Group	B.EO	B.OWL	B.OWLM
k_Bacteria.p_Tenericutes.c_Mollicutes.o_RF39	0.001078	**1.28E-06**	-2.56E-05	0.747381	1.93E-05	0.115488	-0.00127	-0.00088	-0.00119
k_Bacteria.p_Tenericutes.c_Mollicutes.o_RF39.f_	0.001078	**1.28E-06**	-2.56E-05	0.747381	1.93E-05	0.115488	-0.00127	-0.00088	-0.00119
k_Bacteria.p_Tenericutes.c_Mollicutes.o_RF39.f_.g_	0.001078	**1.28E-06**	-2.56E-05	0.747381	1.93E-05	0.115488	-0.00127	-0.00088	-0.00119
k_Bacteria.p_Tenericutes.c_Mollicutes.o_RF39.f_.g_.s_	0.001078	**1.28E-06**	-2.56E-05	0.747381	1.93E-05	0.115488	-0.00127	-0.00088	-0.00119
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium	0.000513	**5.01E-06**	-1.59E-05	0.001107	9.70E-06	0.119983	0.000667	0.000159	0.00022
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium.s_ruminantium	0.000513	**5.01E-06**	-1.59E-05	0.001107	9.70E-06	0.119983	0.000667	0.000159	0.00022
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53	0.001389	**2.00E-05**	-2.72E-05	**5.39E-08**	7.58E-06	**6.72E-05**	0.002456	3.62E-05	0.000418
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_SMB53.s_	0.001389	**2.00E-05**	-2.72E-05	**5.39E-08**	7.58E-06	**6.72E-05**	0.002456	3.62E-05	0.000418
k_Bacteria.p_Firmicutes.Other	0.000949	**3.73E-05**	-1.75E-05	**0.00012**	1.85E-05	0.016494	0.000636	0.000338	0.000707
k_Bacteria.p_Firmicutes.Other.Other	0.000949	**3.73E-05**	-1.75E-05	**0.00012**	1.85E-05	0.016494	0.000636	0.000338	0.000707
k_Bacteria.p_Firmicutes.Other.Other.Other	0.000949	**3.73E-05**	-1.75E-05	**0.00012**	1.85E-05	0.016494	0.000636	0.000338	0.000707
k_Bacteria.p_Firmicutes.Other.Other.Other.Other	0.000949	**3.73E-05**	-1.75E-05	**0.00012**	1.85E-05	0.016494	0.000636	0.000338	0.000707
k_Bacteria.p_Firmicutes.Other.Other.Other.Other.Other	0.000949	**3.73E-05**	-1.75E-05	**0.00012**	1.85E-05	0.016494	0.000636	0.000338	0.000707
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Christensenellaceae	0.000725	**4.87E-05**	7.98E-06	0.021157	2.16E-05	0.205043	-0.00019	-2.90E-05	-0.00026
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Christensenellaceae.g_	0.000725	**4.87E-05**	7.98E-06	0.021157	2.16E-05	0.205043	-0.00019	-2.90E-05	-0.00026
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Christensenellaceae.g_.s_	0.000725	**4.87E-05**	7.98E-06	0.021157	2.16E-05	0.205043	-0.00019	-2.90E-05	-0.00026
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia	0.00185	**7.31E-05**	2.13E-05	0.083285	2.89E-05	0.010434	-0.00111	0.000404	0.000171
k_Bacteria.p_Actinobacteria.c_Coriobacteriia.o_Coriobacteriales.f_Coriobacteriaceae.g_Adlercreutzia.s_	0.00185	**7.31E-05**	2.13E-05	0.083285	2.89E-05	0.010434	-0.00111	0.000404	0.000171
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae	0.00705	0.003656	8.12E-05	0.014004	-6.00E-05	**1.74E-06**	-0.00277	-0.00894	-0.00843
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae.g_Lactococcus	0.00681	0.004863	7.60E-05	0.010361	-6.60E-05	**1.61E-06**	-0.00206	-0.00848	-0.00801
k_Bacteria.p_Firmicutes.c_Bacilli.o_Lactobacillales.f_Streptococcaceae.g_Lactococcus.s_	0.006805	0.004981	7.58E-05	0.010464	-6.63E-05	**1.63E-06**	-0.00205	-0.00847	-0.00801
k_Bacteria.p_Firmicutes.c_Bacilli.Other	0.000372	0.006693	-3.60E-06	**4.13E-08**	1.71E-06	**1.17E-05**	0.000391	-0.0002	-0.00014
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other	0.000372	0.006693	-3.60E-06	**4.13E-08**	1.71E-06	**1.17E-05**	0.000391	-0.0002	-0.00014
k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other	0.000372	0.006693	-3.60E-06	**4.13E-08**	1.71E-06	**1.17E-05**	0.000391	-0.0002	-0.00014

k_Bacteria.p_Firmicutes.c_Bacilli.Other.Other.Other.Other	0.000372	0.006693	-3.60E-06	**4.13E-08**	1.71E-06	**1.17E-05**	0.000391	-0.0002	-0.00014
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_	0.001276	0.029967	-1.38E-05	**3.91E-08**	2.39E-05	**0.000172**	0.001754	0.000113	6.36E-05
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_.s_	0.001276	0.029967	-1.38E-05	**3.91E-08**	2.39E-05	**0.000172**	0.001754	0.000113	6.36E-05
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other	0.005498	0.04851	-2.43E-05	**2.89E-05**	-2.29E-05	**6.24E-05**	0.002967	-0.00234	-0.00165
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae.Other.Other	0.005498	0.04851	-2.43E-05	**2.89E-05**	-2.29E-05	**6.24E-05**	0.002967	-0.00234	-0.00165
k_Bacteria.Other	0.000595	0.071052	-2.00E-06	**1.19E-05**	-2.02E-05	**1.68E-07**	0.000852	-8.65E-05	0.000306
k_Bacteria.Other.Other	0.000595	0.071052	-2.00E-06	**1.19E-05**	-2.02E-05	**1.68E-07**	0.000852	-8.65E-05	0.000306
k_Bacteria.Other.Other.Other	0.000595	0.071052	-2.00E-06	**1.19E-05**	-2.02E-05	**1.68E-07**	0.000852	-8.65E-05	0.000306
k_Bacteria.Other.Other.Other.Other	0.000595	0.071052	-2.00E-06	**1.19E-05**	-2.02E-05	**1.68E-07**	0.000852	-8.65E-05	0.000306
k_Bacteria.Other.Other.Other.Other.Other	0.000595	0.071052	-2.00E-06	**1.19E-05**	-2.02E-05	**1.68E-07**	0.000852	-8.65E-05	0.000306
k_Bacteria.Other.Other.Other.Other.Other.Other	0.000595	0.071052	-2.00E-06	**1.19E-05**	-2.02E-05	**1.68E-07**	0.000852	-8.65E-05	0.000306
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Ruminococcaceae	0.116528	0.075389	0.000404	0.353825	-0.0012	**0.000118**	0.027685	-0.02768	-0.03497
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae	0.000352	0.078335	1.88E-06	0.70151	-3.59E-06	**0.000202**	-8.50E-05	-0.00026	-0.00031
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae.g_Dehalobacterium	0.000352	0.078335	1.88E-06	0.70151	-3.59E-06	**0.000202**	-8.50E-05	-0.00026	-0.00031
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Dehalobacteriaceae.g_Dehalobacterium.s_	0.000352	0.078335	1.88E-06	0.70151	-3.59E-06	**0.000202**	-8.50E-05	-0.00026	-0.00031
k_Bacteria.p_Firmicutes.c_Erysipelotrichi	0.306578	0.177167	-0.00147	0.34803	0.008521	**1.64E-05**	-0.21104	0.153866	0.1081
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales	0.306578	0.177167	-0.00147	0.34803	0.008521	**1.64E-05**	-0.21104	0.153866	0.1081
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae	0.306578	0.177167	-0.00147	0.34803	0.008521	**1.64E-05**	-0.21104	0.153866	0.1081
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum	0.301559	0.235562	-0.00136	0.36987	0.008552	**2.03E-05**	-0.21091	0.152765	0.104212
k_Bacteria.p_Firmicutes.c_Erysipelotrichi.o_Erysipelotrichales.f_Erysipelotrichaceae.g_Allobaculum.s_	0.301559	0.235562	-0.00136	0.36987	0.008552	**2.03E-05**	-0.21091	0.152765	0.104212
k_Bacteria.p_Firmicutes.c_Clostridia	0.560382	0.260467	0.001283	0.38609	-0.00769	**0.000183**	0.219705	-0.09783	-0.05717
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales	0.560382	0.260467	0.001283	0.38609	-0.00769	**0.000183**	0.219705	-0.09783	-0.05717
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae.	0.001581	0.705011	1.04E-06	0.614855	8.87E-06	**0.000109**	-0.00087	-0.00098	-0.00151
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_	0.001276	0.98419	-6.69E-07	0.486644	9.43E-06	**0.000179**	-0.00069	-0.00081	-0.00133
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_.Mogibacteriaceae..g_.s_	0.001276	0.98419	-6.69E-07	0.486644	9.43E-06	**0.000179**	-0.00069	-0.00081	-0.00133

Figure 25. Scatter plots of *Adlercreutzia* genus: A. all groups; B. EO only; C. OWLM only; and D. OWL only at four time points (T1 – week 61; T2 – week 75; T5 – week 88; T8 – week 102).

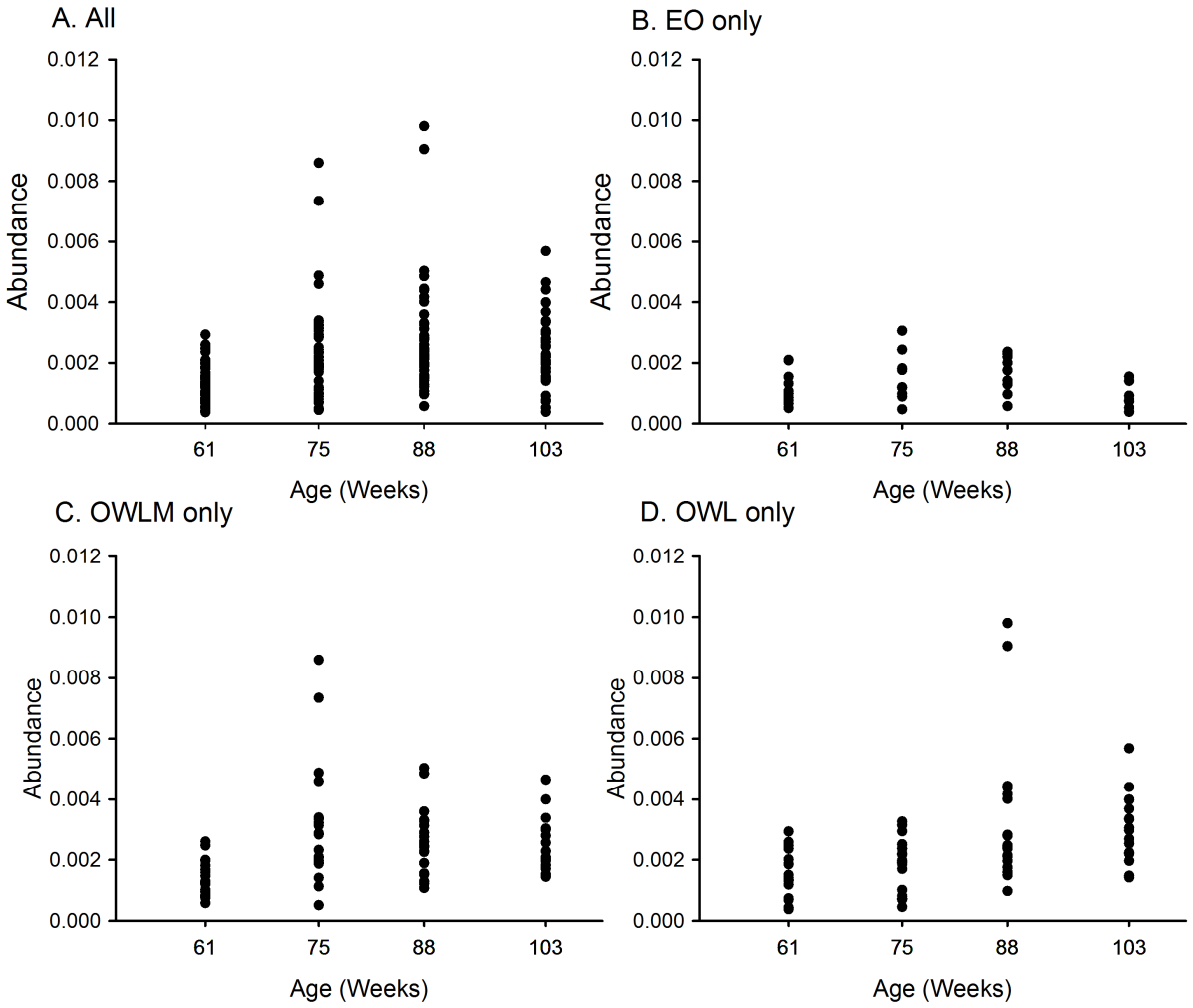
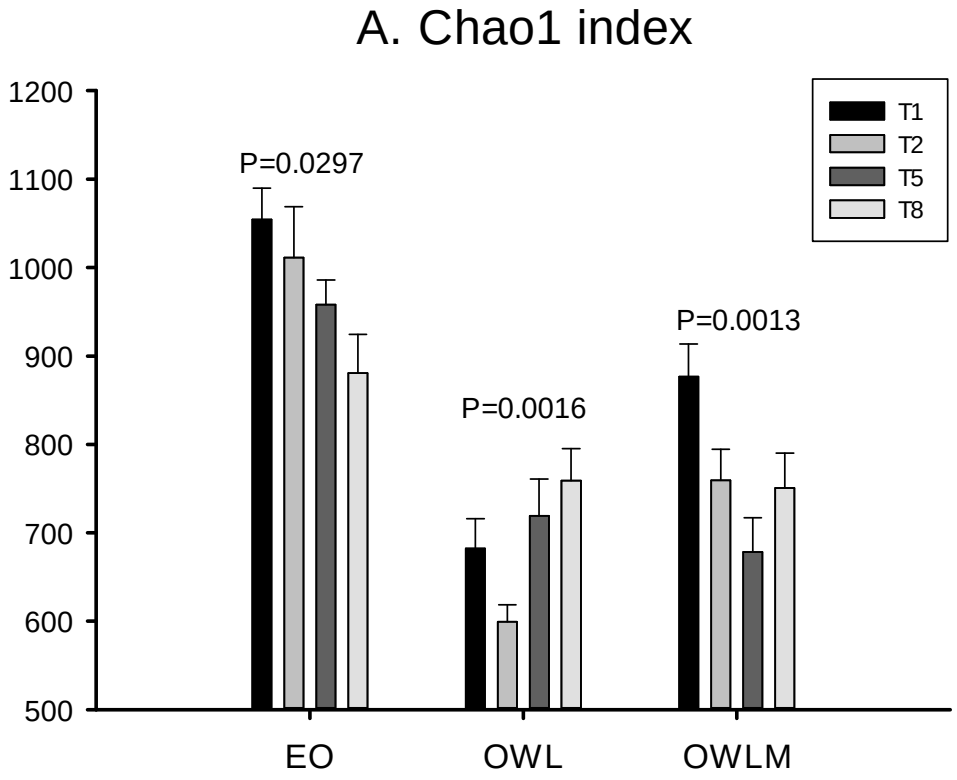
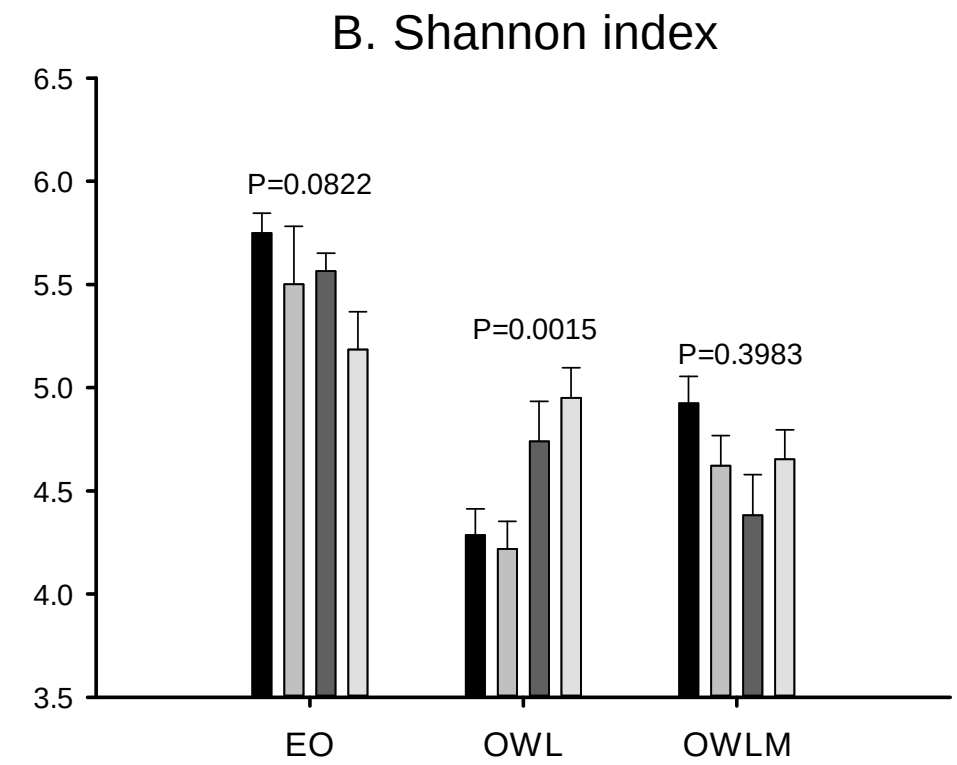


Figure 26. Alpha diversity comparisons of the four time points within group (T1 –week 61; T2 week 75; T5 week 88; T8 – week 102).





Discussion

Aging is accompanied by changes in gastrointestinal tract physiology and the immune system, in addition to changes in diet and lifestyle [118, 119], all of which could further cause the gut microbiota changes [48]. Generally, the human intestinal microbiota is relatively stable in healthy adults [120]; however, perturbations in the intestinal microbiota were identified in elderly individuals [17, 93, 121]. It was found that there was an age-related reduction of the abundance of genes in pathways involved in SCFA production [122], which might suggest the bacteria related to SCFA production could be diminishing with age. Here, we monitored the mice gut microbiome from adulthood to old age to identify microbial composition of healthy aged mice under *ad libitum* feeding or caloric restriction with the same diet.

For the within-group diversity, interestingly, the EO group had both the highest total number of bacterial species (as indicated by the Chao1 index) and evenness of distribution (as indicated by the Shannon index). In the Chao1 index, OWLM had a lower score than EO, with OWL being the lowest, which is exactly the same order in their body weight or food intake. For the Shannon index, although there was no significant difference between OWL and OWLM, they were both significantly lower than EO, with OWLM having a slightly higher value than OWL. This suggests that sustainable lower food intake or body weight could reduce the microbial species number and evenness of distribution. The insignificance between time points (from week 61 to week 103) suggests that aging might not influence the number of species and the evenness of distribution in gut microbiome communities.

Only one genus of bacterium was found to be consistently significantly different between time point 1 and the three other time points: *Adlercreutzi*a. This is a bacterium with very low abundance (less than 0.2%). Its metabolic function has not been clearly elucidated other than to convert daidzein to equol. More OTUs were significantly correlated with animal age than the time points, such as *Clostridium.ruminantium* and the *Christensenellacea*e family. All of these OTUs had abundances less than 0.2%, on average. Despite the significant increase with aging for these three mice groups under fixed feeding, it is unknown yet how this special low-abundance bacterium is related to biological aging.

We did not look at the compositions of gut microbiome at the extremes of life; however, these long-term microbial changes in long-term healthy adult mice showed that the bacteria were relatively stable (except for a few low-abundant bacterial lineages) under fixed feeding, which is in agreement with the literature [17, 123].

Hypothesis 3b.

Baseline Fecal Composition (or Specific Strains of Bacteria) Will Be the Same at Baseline between Short-lived and Long-lived Mice

Results

A total of 80 samples were selected (EO: 10 long-lived, 10 short-lived; OWLM: 20 long-lived, 10 short-lived; WC: 20 long-lived, 10 short-lived). OWL was not included in this analysis because, at the time of the study design, there was not enough mortality in the OWL group. **Figure 27** below shows the survival curves for all samples and each group stratified by long-lived or short-lived status. The results of survival analyses were

shown in **Table 19**. Whether combined or separated, the long-lived groups had a significantly longer life span than the short-lived groups. For the basic characteristics at the time of sample collection, there was no significant difference between the long-lived group and the short-lived group in average daily food intake (g/day) (P=0.7033), body weight (P=0.3860), fat mass (P=0.5124) or fat-free mass (P=8108), indicating that these baseline characteristics might not be predictive of future lifespan.

As shown in **Figure 28** below, for all three groups combined, the Chao1 alpha diversity of the long-lived group was significantly higher than that of the short-lived group (P=0.0041), while the Shannon index was not significantly different (P=0.6899). As for the beta diversity, the two groups were clearly separated from each other in the unweighted UniFrac PCoA plot, but not well separated in the Bray-Curtis or weighted UniFrac PCoA plots. This indicates that the distributions were relatively similar between the two groups and that the OTUs causing phylogenetic differences were primarily low-abundant ones.

The first regression model (OTU = short-lived/long-lived + group) aimed to find the differences in specific OTUs at various levels controlling for group differences. As shown in **Table 20**, 17 OTUs at various levels (six bacterial lineages) reached statistic significance after Bonferroni correction. Most of these OTUs had very low average abundance (from 0.04% to 0.15%). The scatter distributions of six representative OTUs were shown in **Figure 29** (categorized by short-lived/long-lived and group), and from this figure, we can see that the abundances of these OTUs were lower in the short-lived groups than the long-lived groups. Of them, there were two lineages of bacteria that were unnamed or unidentified under bacteria kingdom and *Firmicutes* phylum, respectively.

The other identified significant OTUs included the *Clostridium.celatum* species, the *Bacteroidaceae* family, the *Bacteroides* genus, the *Clostridium* genus, the *Clostridium.ruminantium* species and the *Blautia* genus (under the *Lachnospiraceae* family). In addition, the OWLM group had significantly different proportions of the *Bacteroidaceae* family, the *Bacteroides* genus and the *Blautia* genus when compared to the EO group. There was no group difference between the EO and WC groups in these OTUs.

Another regression model (OTU = lifespan + group) treated lifespan as a continuous variable while controlling for groups (**Table 21**). There were fewer significant OTUs reaching significance at this time. The first unnamed lineages under the *Firmicutes* phylum also appeared in the previous model. The only OTU that was significantly correlated with lifespan except the unnamed lineage was *Clostridium.celatum*, which also reached significance in the previous model. Spearman association tests revealed significant associations between the unnamed *Firmicutues.other* class abundance and lifespan (Rho=0.48744, P<0.0001) and between *Clostridium.celatum* abundance and lifespan (Rho=0.34216, P=0.0020). Interestingly, when the association was tested for the short-lived and long-lived groups separately, none of these pairs reached significance: *Firmicutues.other* vs. short-lived lifespan (Rho=0.00200, P=0.9916), *Firmicutues.other* vs. long-lived lifespan (Rho=0.01454, P=0.9210), *Clostridium.celatum* vs. short-lived lifespan (Rho=0.15510, P=0.4151) or *Clostridium.celatum* vs. long-lived lifespan (Rho=0.00765, P=0.9584).

Although after Bonferroni correction, the *Bacteroidaceae* family did not reach targeted P-value (0.05/145=0.0003), an interesting phenomenon was that close to half of

these samples had zero abundance in this *Bacteroidaceae* family. Survival analysis was used to compare the samples with presence or absence of the *Bacteroidaceae* family and showed that there were significant differences in survival analysis between these two categories in all three groups together, OWLM only and WC only, but not in EO only (see **Figure 30** and **Table 22**).

Table 19. Survival analysis for the long-lived and short-lived samples selected (all: n=30/50, short-lived/long-lived; EO: n=10/10; OWLM: n=10/20; WC: n=10/20).

	Test	Chi-Square	DF	Pr > Chi-Square
ALL	Log-Rank	99.6030	1	<0.0001
	Wilcoxon	85.9396	1	<0.0001
EO	Log-Rank	13.0017	1	0.0003
	Wilcoxon	10.3848	1	0.0013
OWLM	Log-Rank	40.7188	1	<0.0001
	Wilcoxon	36.3636	1	<0.0001
WC	Log-Rank	40.7188	1	<0.0001
	Wilcoxon	36.3636	1	<0.0001

Figure 27. Survival curves for the long-lived and short-lived samples selected: A. All three groups combined (n=30/50, short-lived/long-lived); B. EO group only (n=10/10); C. OWLM group only (n=10/20); D. WC group only (n=10/20).

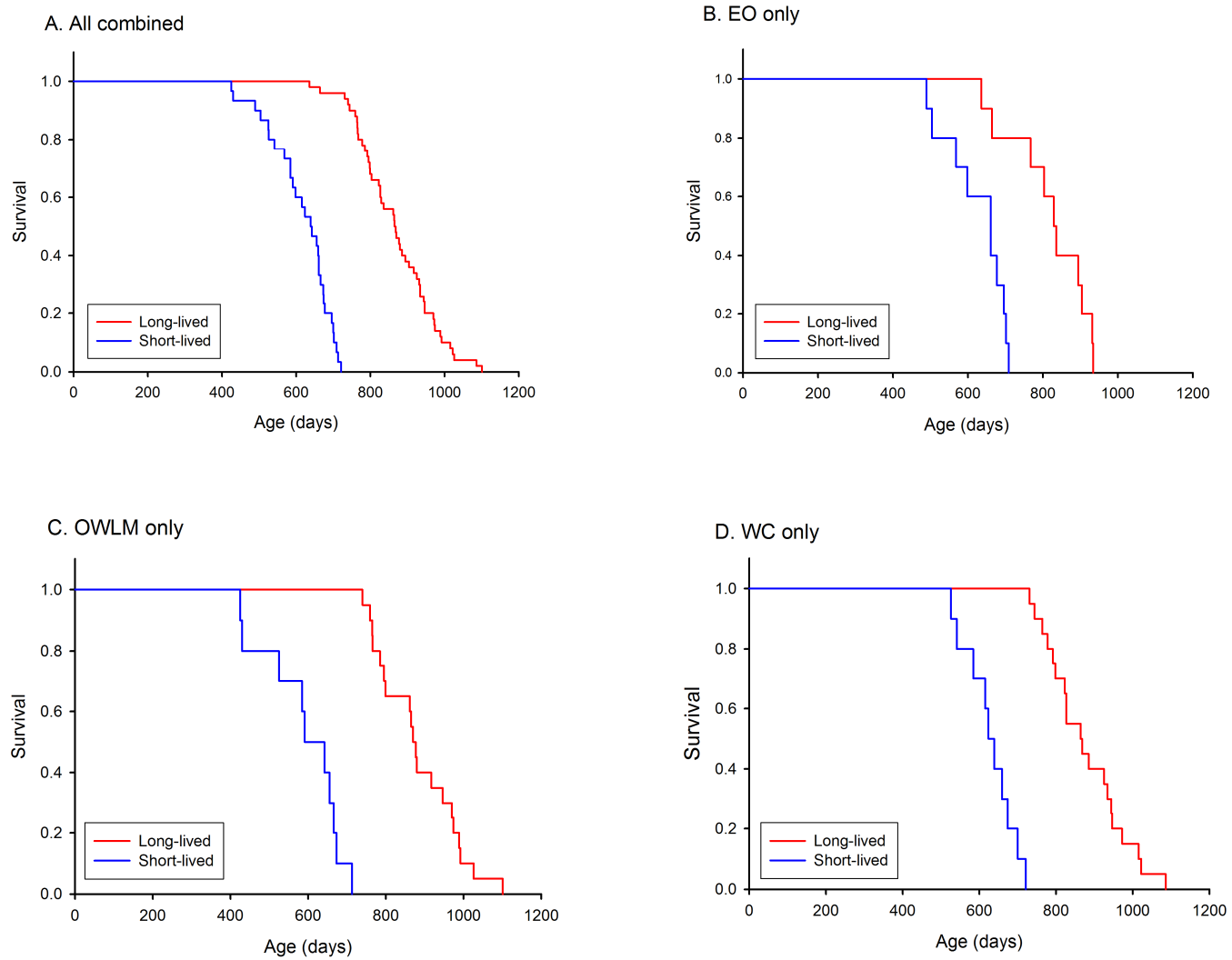


Figure 28. Alpha diversity and beta diversity for the short-lived and long-lived groups with all three groups combined (short-lived: n=30; long-lived: n=50; all samples were collected at pre-randomization).

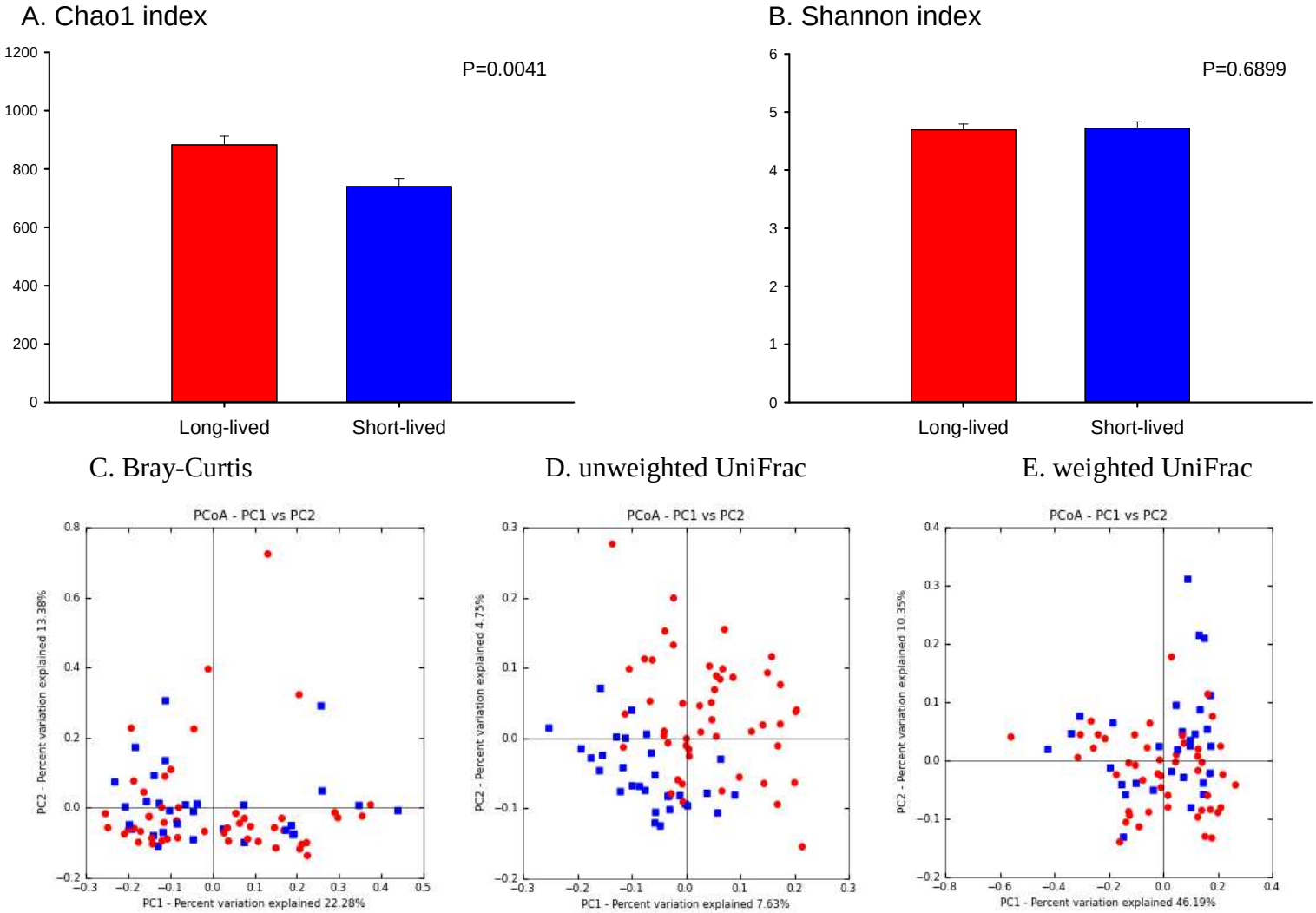


Table 20. Significantly different OTUs between long-lived and short-lived groups after controlling for group differences (EO: n=10/10, short-lived/long-lived; OWLM: n=10/20; WC: n=10/20; samples were collected at pre-randomization/week 44).

	Abundance Long-lived		Abundance Short-lived		Dead/Alive P-value	Short/Long Beta	OWLM P-value	OWLM Beta	WC P-value	WC Beta
k_Bacteria.p_Firmicutes.Other	0.15%	0.01%	0.06%	0.01%	**8.63E-07**	-0.00095	0.730349	7.58E-05	0.210815	-0.00028
k_Bacteria.p_Firmicutes.Other.Other	0.15%	0.01%	0.06%	0.01%	**8.63E-07**	-0.00095	0.730349	7.58E-05	0.210815	-0.00028
k_Bacteria.p_Firmicutes.Other.Other.Other	0.15%	0.01%	0.06%	0.01%	**8.63E-07**	-0.00095	0.730349	7.58E-05	0.210815	-0.00028
k_Bacteria.p_Firmicutes.Other.Other.Other.Other	0.15%	0.01%	0.06%	0.01%	**8.63E-07**	-0.00095	0.730349	7.58E-05	0.210815	-0.00028
k_Bacteria.p_Firmicutes.Other.Other.Other.Other.Other	0.15%	0.01%	0.06%	0.01%	**8.63E-07**	-0.00095	0.730349	7.58E-05	0.210815	-0.00028
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_Clostridium.s_celatum	0.04%	0.00%	0.02%	0.00%	**5.73E-06**	-0.00027	0.148649	-9.91E-05	0.022561	-0.00016
k_Bacteria.p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Bacteroidaceae	0.08%	0.01%	0.00%	0.00%	**4.01E-05**	-0.00068	**1.11E-05**	0.000913	0.495757	0.000133
k_Bacteria.p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Bacteroidaceae.g_Bacteroides	0.08%	0.01%	0.00%	0.00%	**4.01E-05**	-0.00068	**1.11E-05**	0.000913	0.495757	0.000133
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium	0.06%	0.01%	0.02%	0.00%	**4.27E-05**	-0.00039	0.011734	-0.00029	0.03136	-0.00025
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium.s_ruminantium	0.06%	0.01%	0.02%	0.00%	**4.27E-05**	-0.00039	0.011734	-0.00029	0.03136	-0.00025
k_Bacteria.Other	0.09%	0.01%	0.05%	0.01%	**4.32E-05**	-0.00049	0.000939	-0.00048	0.091512	-0.00024
k_Bacteria.Other.Other	0.09%	0.01%	0.05%	0.01%	**4.32E-05**	-0.00049	0.000939	-0.00048	0.091512	-0.00024
k_Bacteria.Other.Other.Other	0.09%	0.01%	0.05%	0.01%	**4.32E-05**	-0.00049	0.000939	-0.00048	0.091512	-0.00024
k_Bacteria.Other.Other.Other.Other	0.09%	0.01%	0.05%	0.01%	**4.32E-05**	-0.00049	0.000939	-0.00048	0.091512	-0.00024
k_Bacteria.Other.Other.Other.Other.Other	0.09%	0.01%	0.05%	0.01%	**4.32E-05**	-0.00049	0.000939	-0.00048	0.091512	-0.00024
k_Bacteria.Other.Other.Other.Other.Other.Other	0.09%	0.01%	0.05%	0.01%	**4.32E-05**	-0.00049	0.000939	-0.00048	0.091512	-0.00024
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_Blautia	0.08%	0.02%	0.01%	0.00%	**0.000189**	-0.00069	**4.82E-05**	0.000939	0.621727	0.000108

Table 21. OTUs that correlated with lifespan after controlling for group differences (EO: n=20; OWLM: n=30; WC: n=30; samples were collected at pre-randomization/week 44).

	Average Abundance		Lifespan P-value	Lifespan Beta	OWLM P-value	OWLM Beta	WC P-value	WC Beta
k_Bacteria.p_Firmicutes.Other	0.11%	0.01%	**4.46E-05**	2.52E-06	0.747227	7.49E-05	0.196476	-0.0003
k_Bacteria.p_Firmicutes.Other.Other	0.11%	0.01%	**4.46E-05**	2.52E-06	0.747227	7.49E-05	0.196476	-0.0003
k_Bacteria.p_Firmicutes.Other.Other.Other	0.11%	0.01%	**4.46E-05**	2.52E-06	0.747227	7.49E-05	0.196476	-0.0003
k_Bacteria.p_Firmicutes.Other.Other.Other.Other	0.11%	0.01%	**4.46E-05**	2.52E-06	0.747227	7.49E-05	0.196476	-0.0003
k_Bacteria.p_Firmicutes.Other.Other.Other.Other.Other	0.11%	0.01%	**4.46E-05**	2.52E-06	0.747227	7.49E-05	0.196476	-0.0003
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_Clostridium.s_celatum	0.03%	0.00%	**0.000181**	7.05E-07	0.16859	-9.90E-05	0.023648	-0.00017
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_	0.29%	0.04%	0.000495	8.04E-06	0.57492	-0.00049	0.240548	-0.00105
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Clostridiaceae.g_.s_	0.29%	0.04%	0.000495	8.04E-06	0.57492	-0.00049	0.240548	-0.00105
k_Bacteria.p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Bacteroidaceae	0.05%	0.01%	0.0006	1.81E-06	**2.16E-05**	0.000913	0.573961	0.000115
k_Bacteria.p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Bacteroidaceae.g_Bacteroides	0.05%	0.01%	0.0006	1.81E-06	**2.16E-05**	0.000913	0.573961	0.000115
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium	0.05%	0.00%	0.000839	1.01E-06	0.015782	-0.00029	0.032681	-0.00025
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Peptostreptococcaceae.g_Clostridium.s_ruminantium	0.05%	0.00%	0.000839	1.01E-06	0.015782	-0.00029	0.032681	-0.00025
k_Bacteria.p_Firmicutes.c_Clostridia.o_Clostridiales.f_Lachnospiraceae.g_Blautia	0.05%	0.01%	0.001063	1.91E-06	**7.77E-05**	0.000934	0.709887	8.41E-05

Figure 29. Scatter distribution of the significantly different OTU lineages in short-lived/long-lived comparisons. (Blue – short-lived mice; red – long-lived mice; EO: n=10/10, short-lived/long-lived; OWLM: n=10/20; WC: n=10/20; samples were collected at pre-randomization/week 44.)

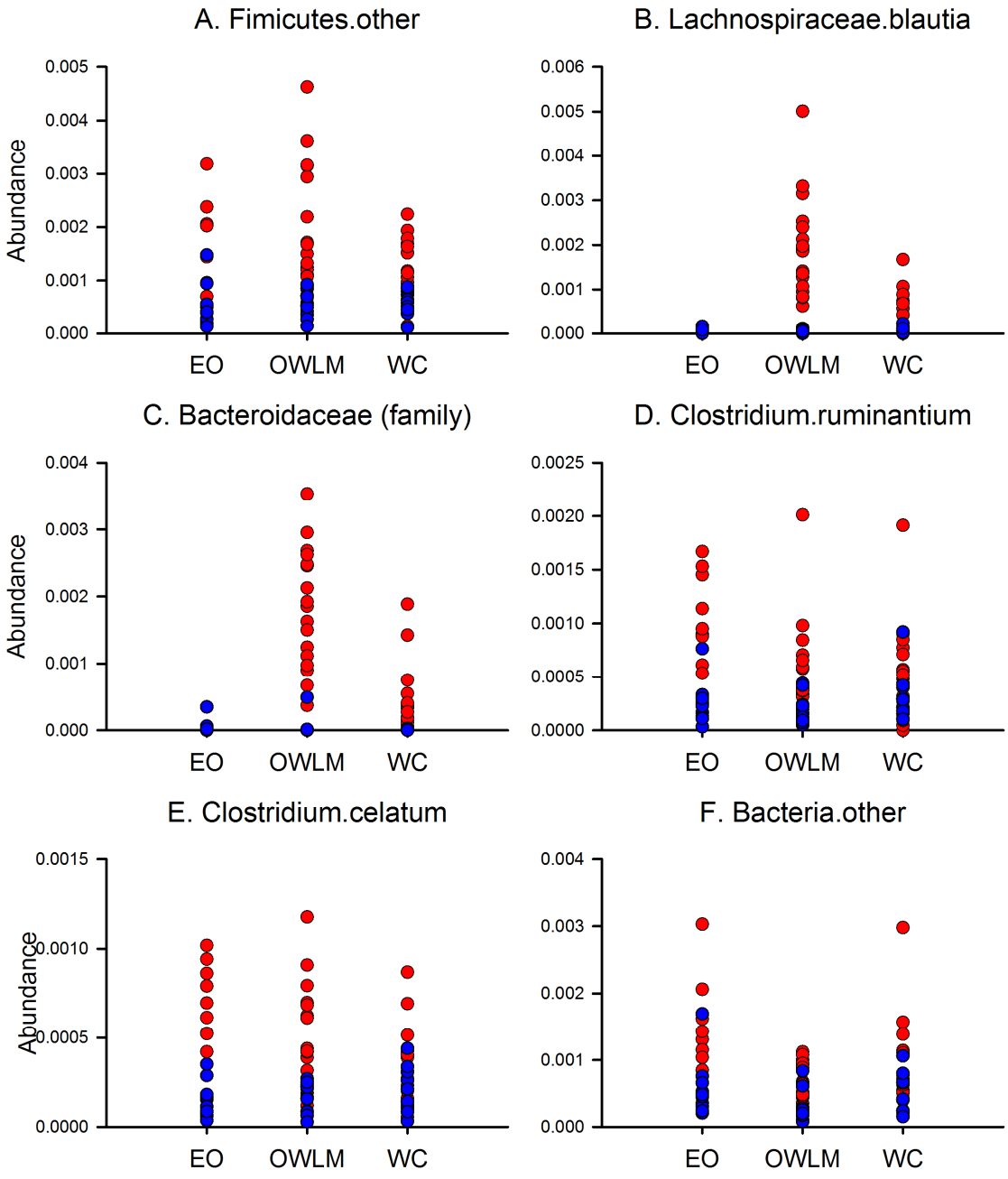


Figure 30. Survival curves for the groups with the presence or absence of *Bacteroidaceae* family: A. All three groups combined (n=31/48, absence/presence); B. EO group only (n=13/7); C. OWLM group only (n=10/20); D. WC group only (n=8/21) (samples were collected at pre-randomization/week 44).

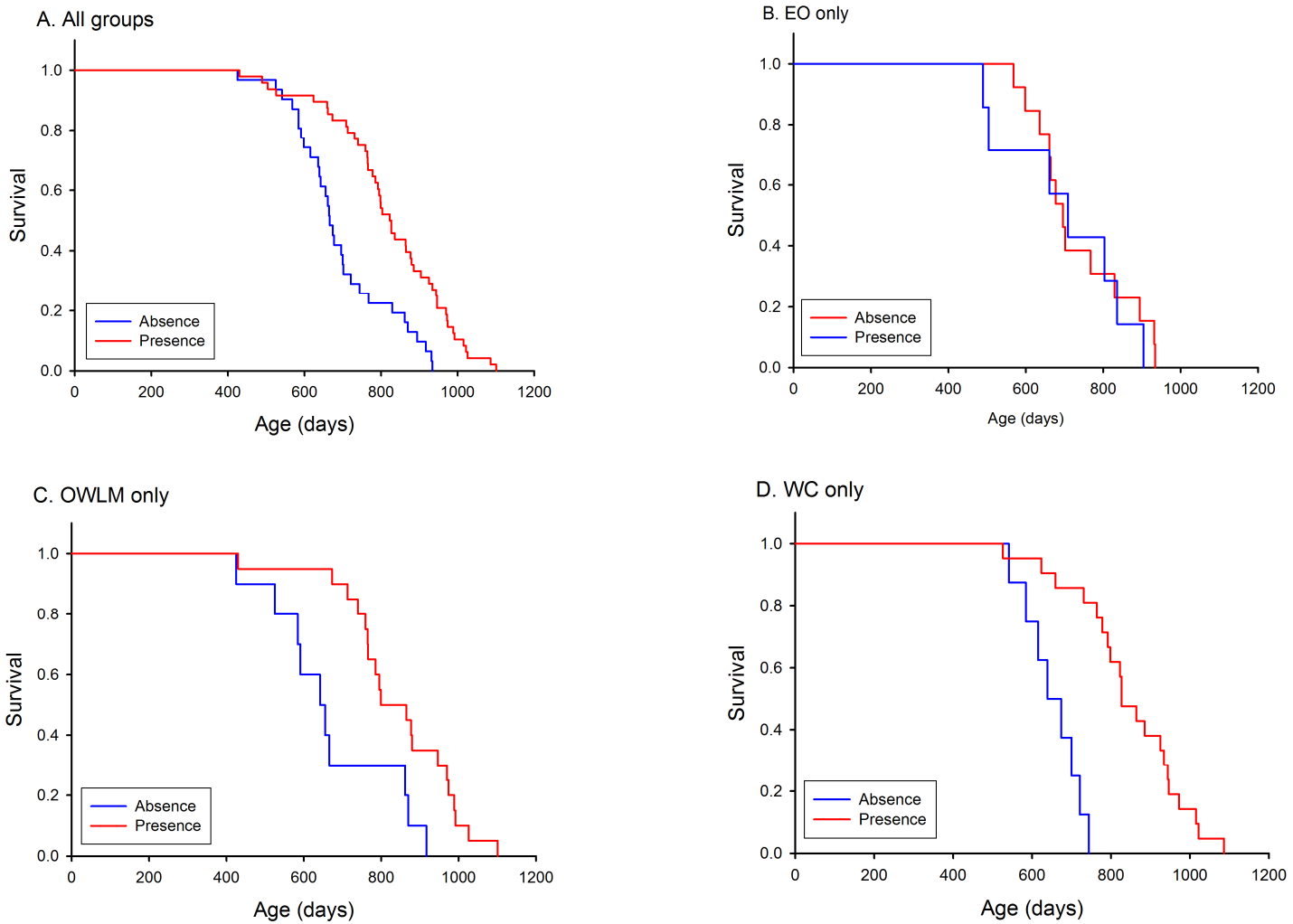


Table 22. Survival analysis for the mice with the presence and absence of the *Bacteroidaceae* family (for EO, OWLM and WC combined and separately; for EO n=13/7, absence/presence; for OWLM, n=10/20, absence/presence; for WC, n=8/21, absence/presence).

	Test	Chi-Square	DF	Prob>ChiSq
All	Log-Rank	17.8474	1	<0.0001*
	Wilcoxon	16.7354	1	<0.0001*
EO	Log-Rank	0.1662	1	0.6835
	Wilcoxon	0.1019	1	0.7495
OWLM	Log-Rank	8.1455	1	0.0043*
	Wilcoxon	8.8999	1	0.0029*
WC	Log-Rank	19.9510	1	<.0001*
	Wilcoxon	16.5000	1	<.0001*

Discussion

Previous studies have shown that centenarians had a distinct microbiome composition with certain bacteria over-represented when compared to elder people or young people [48, 122]. Zhang *et al.* [124] found that calorie restriction enriches phylotypes positively correlated with lifespan (for example, the genus *Lactobacillus*) and reduces phylotypes negatively correlated with lifespan, which further suggests that calorie restriction can establish a structurally balanced architecture of gut microbiota that may exert a health benefit to the host via reduction of antigen load from the gut. In this study, we focused on the relationship between microbiome compositions in mice at a younger age (10 months old) and subsequent lifespan.

The long-lived group had a significantly greater number of bacterial species than the short-lived group, which suggests that having a more diverse bacterial community at a younger age, regardless of the dietary treatment, might be related to longevity later. Nevertheless, from our preliminary survival analysis, the EO group has the shortest lifespan, while other groups have longer lifespan. But in our previous results, on the contrary, the EO group had the greatest number of species compared to OWLM and OWL groups. Studies have also found that subjects with diseased status had decreased diversity in fecal microbiome [125]. This contradiction in the Chao1 index further indicates that the total number of species is not the simple cause for longer lifespan. We found that there were several OTUs that were significantly different in abundance between the long-lived and short-lived mice: *Clostridium.celatum*, *Bacteroides genus*, *Clostridium.ruminantium* and *Blautia genus*. All of these OTUs had a relatively low proportion; however, they were generally higher in the long-lived mice than the short-

lived mice. Spearman correlations between these bacteria and lifespan revealed positive statistical significance: *Firmicutues.other* (rho=0.48744, P<0.0001), *Clostridium.celatum* (rho=0.34216, P=0.0020), *Clostridium.ruminantium* (rho=0.31671, P=0.0045), and *Blautia* (rho=0.41684, P=0.0001). This suggests that these bacteria might play a role in promoting longer lifespan, despite their low abundances. A literature search revealed no obvious connection between the functions of these bacteria and health promotion. *Blautia* was recently isolated from human feces and found to be able to produce acid from various carbohydrates and contains cellular straight-chain saturated and mono-unsaturated fatty acids [126]; *Clostridium.celatum* was found to reduce sulfite and nitrite and produce urease [127]. Clearly further studies are needed to investigate their potential roles in health maintenance or life promotion.

In addition, for the *Bacteroides* family (and genus), the presence of these bacteria is related to longer lifespan in the moderately caloric restricted OWLM group and weight cycled WC group, but not in the *ad libitum*–fed EO group. To date and to our knowledge, this is the first time that the presence or abundance of one specific bacterium is shown to be associated with shorter/longer lifespan. Although we cannot directly infer causality between these bacteria and lifespan, this might imply that the lifespan potential might be "pre-determined" by certain factors related to gut microbiome (for example, host genetic background, individual immune system, etc.) in early life stages.

GENERAL DISCUSSION AND CONCLUSION

This study investigated the changes of gut microbiome from multiple aspects: body weight variation, digestive efficiency, different levels of calorie restriction, chronic body weight or food intake changes, restorability after weight cycles, aging under fixed feeding and longevity prediction. Due to the uniqueness of the mouse longevity project, most of the hypotheses were tested for the first time. Additional strengths of this study are that all the animals were the same inbred strain and received exactly the same type of diet, which could eliminate many confounding factors.

The fact that gut microbiome changes after diet alterations suggests there is a need to control for dietary variation when evaluating the composition of the gut microbiome [6]. Here all the animal subjects received the exact same diet throughout the study, therefore we precluded the effects of different diet components on gut microbiome composition. We found that the gut microbiome composition, similar to body weight, could have great variations in the same inbred strain of mice. This variation is hardly explained by the simple dietary factors and, more possibly, could be related to the subtle genetic differences inherited from maternal parts or early life exposures to different environmental factors. We identified a number of bacteria related to digestive efficiency, and the relationship between these bacterial populations and fecal energy content is not simply the extraction of normally indigestible energy. We found certain bacteria that are dose dependent on the levels of calorie restriction compared to *ad libitum* feeding. We

also monitored the long-term bacterial changes during ongoing weight loss or food restriction but found the changes occurred only in a few low-abundant bacteria. Furthermore, we were able to detect the microbial composition differences after weight loss and regain cycles, which implies that weight cycling is not simply regaining the lost mass but more likely could influence the bacterial community and even the hosts. We also confirmed the literature that gut microbiome are relatively stable in adult under fixed feeding, by following up the gut microbiome compositions over 40 weeks in adult mice. Last, an intriguing finding of this study is that having some low-abundant bacteria at a younger age is associated with longevity, and the absence or presence of some bacteria at a younger age could predict lifespan.

Environmental factors and host genetics clearly interact to control the acquisition and to maintain the stability of gut microbiota [79]. Additionally, epigenetic modification could be one of the reasons explaining the variability in adiposity and microbial compositions within this study. It was suggested that there is broad variation in the importance of heritable influences and environmental or stochastic variation to DNA methylation [128]. Studies with monozygotic twins have been used to test this hypothesis. Previous studies have found that various tissues of monozygotic twins already show differences in DNA methylation at birth [129, 130]. Another study found that differences between twins in average genome-wide DNA methylation and total histone acetylation levels increase with age [131], which indicates the postnatal epigenetic changes must be different even between twins. Therefore in this study the individual variations in body weight of these same strain of inbred C57BL/6J mice could be caused by the early environmental and stochastic influences. Additionally, recent

studies suggest that there could be correlations between epigenetics, caloric restriction and organismal longevity [132]. On the other hand, microbial metabolites can influence epigenetics by altering the pool of compounds used for modification or by directly inhibiting enzymes involved in epigenetic pathways [133]. Studies have shown that the gut microbiota in obesity and type 2 diabetes affect the epigenetic regulation of genes, which may involve short chain fatty acids signaling pathways in the gut epithelium [134]. Consequently there must be an interaction between the host genetic or epigenetic factors and their intestinal bacterial community, and taken together this interaction determines the unique individual microbial compositions. Nevertheless, with these variations, there were still uniformities in the changes of gut microbiome compositions with different dietary treatments in this study.

Short chain fatty acids (SCFAs) are produced by microbiota in the colon and the distal small intestine from resistant starch, dietary fiber, and other low-digestible polysaccharides in fermentation process [118]. Those SCFAs are readily absorbed by coloncytes as energy sources, in addition to their roles as signaling molecules. Supplementation of SCFA in mice showed that butyrate, propionate and acetate all protected against diet-induced obesity and insulin resistance, and that butyrate and propionate induced gut hormones and reduce food intake [135]. Butyrate supplementation also enhanced adaptive thermogenesis and fatty acid oxidation in high-fat diet fed mice, with increased mitochondrial function and biogenesis in skeletal muscle and brown fat [136]. Although we did not measure SCFA concentration changes in this project, previous studies showed that SCFA concentrations were remarkably reduced by food restriction [137]. Of these SCFA changes during food restriction, butyrate had the

greatest reduction compared to other SCFAs [138]. It is worthwhile to keep investigating the health consequences of these short-chain fatty acids and once affirmed, supplementation of specific SCFA might be effective in promoting health status, similarly to the commonly used fish oil (or DHA/EPA) supplementation in maintaining cardiovascular health.

Life-long dietary restriction on both conventional rats and germ-free rats [139] demonstrated that germ-free rats had slightly longer lifespan than their conventional counterparts, and there was no additional effect on lifespan in germ-free rats when food intake was restricted, which contrasts to the life-extending effects of calorie restriction in conventional rats. It was proposed that the life extension in germ-free rats may be due to a natural dietary restriction in the germ-free state. However, as discussed in the study, there were two housing variables - stress from individual housing in the restricted rats and stress from crowding in the ad libitum rats - may have influenced the outcome of the study. Nevertheless, similar observations were later affirmed in mice models [140]. Furthermore, the life extension in germ-free rats is not related to differences in endocrine function [141]. This may be due to the reduced food intake and smaller body weight of germ-free rats, which may be mediated by the absence of energy sources contributed by gut microbiome. However, while the *ad libitum* fed conventional rats were heavier than germ-free counterparts (510g vs. 435g), surprisingly, the dietary restricted germ-free rats were significantly heavier than the dietary restricted conventional rat (340g vs. 300g) [142] when the same amount of food was consumed. This is contradictory to current belief that gut microbiome help to extract indigestible energy from food into short chain fatty acids, which is subsequently available to the hosts and contributes to obesity [1].

Recent studies proposed that the mechanism underlying the fat increase in conventionalization of germ-free mice was the suppressed intestinal expression of fasting-induced adipose factor (Fiaf), which is a circulating lipoprotein lipase leading to triglyceride accumulation in adipocytes [143]. Gut microbiota can directly or indirectly modulate gut motility, alter secretion of gut hormones, gut permeability and immune function. For humans, it is not practical or possible to maintain a 'germ-free' status, thus identifying those 'good' or 'life-promoting' bacteria and boosting their abundance (for example the *Bacteroidaceae* family identified in this study), and/or eliminating those 'bad' or 'life-diminishing' bacteria, could be a potential way of lengthening lifespan.

Nevertheless, there are also some limitations in this study. First, because this study was conceived after the initiation of the mice longevity project, the samples were not collected at baseline at 8 weeks of age, before all animals received a high-fat diet. Second, since this was an ongoing longevity study, we were unable to measure biochemical or histological parameters, nor could we collect cecal contents to determine the cecal microbiome composition, because there could be differences between cecal and fecal microbiome compositions. Third, although the mice were singly housed in a specific pathogen-free facility, their intestinal bacteria might be influenced by sharing the same facility and consuming a diet that was not radiated. Fourth, for hypothesis 3b, since the animals were selected based on their survival status at 104 weeks of age, there might be a selection bias, which might not be representative of all mice within each group. Additionally, the group randomizations in this study were based on body weight and not microbiome compositions, and there was no intervention designed to test the actual functions of specific strains of bacteria; the “role” presented in this study is more

"correlative" or "observational" rather than "causative." The exact sequences from separate reactions are not the same due to the 16S PCR and variation in the DNA that is on the Illumina chip. Last, bioinformatics processing to generate diversities and bacterial proportions is, to some extent, dependent on the sample sizes included.

In conclusion, the results of this study contribute extensive valuable information to the current literature on the microbiome changes in diet-induced obesity, calorie restriction and aging. These results could serve as a basis for future interventional studies to investigate the roles of gut microbiome in obesity and aging, and subsequent clinical applications to obesity treatment and longevity promotion.

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APPENDIX

A. INSTITUTIONAL REVIEW BOARD APPROVAL

Admission to Candidacy
Research Compliance Verification Form

Instructions
Complete this form, including all applicable forms and the signatures of the student, the student's advisor, and the Graduate Program Director. For research approval forms, contact the Institutional Review Board (IRB) (http://www.uab.edu/irb or 934-3789), or the Institutional Animal Care and Use Committee (IACUC) (http://www.uab.edu/iacuc or 934-7692).

Human Subjects
The University of Alabama at Birmingham defines a human subject as not only a living human being, but also human tissue, blood samples, pathology or diagnostic specimens, study of medical records, observation of public behavior, and all questionnaires or surveys.

Does the research proposed by the student involve human subjects? ☐ Yes (continue below) ☒ No
This research is:
Approved _____ Date _____

IRB Protocol No. _____

Attach a copy of your IRB approval. Your own name must appear on the original approval or on an attached amendment.

Animal Subjects
The University of Alabama at Birmingham defines a laboratory animal as any vertebrate animal (e.g., traditional laboratory animals, farm animals, wildlife, and aquatic animals) and certain higher invertebrate animals used in research, teaching, or testing at UAB or sponsored through UAB but conducted off-site (i.e., field research or at collaborating institutions, etc.).

Does the research proposed by the student involve animal subjects? ☒ Yes (continue below) ☐ No

This research is:
Approved YES Date 7-27-12

IACUC Protocol No. 120708909

Attach a copy of your IACUC Notice of Approval, showing your research subject and the animal project number. If your own name does not appear on the Notice of Approval, take this form to the IACUC office for verification of approval.

The IACUC office verifies that Yongbin Yang (name of candidate) is covered under the attached approval.

Signature of IACUC representative _____ Date: 5-31-13

NOTE: The student's advisor, the student, and the Graduate Program Director agree that no research will be initiated until an application is submitted for review and approved by the appropriate review boards (IRB and/or IACUC) if the proposed thesis or dissertation project requires approval. If approval already exists, this student's name must be added to the existing protocol before candidacy will be approved by the Graduate School. It is the responsibility of the student's advisor and the student to comply with federal and UAB regulations associated with this research. Documentation of continuous, appropriate approval will be required before degree conferral; all required IRB and/or IACUC approvals must be current at the time final versions of theses or dissertations are submitted to the Graduate School.

Student's Signature	Nutrition Sciences Dept.	05/31/2013 Date
Signature of Student's Advisor	Nutrition Sciences Dept.	05/31/2013 Date
Graduate Program Director	Nutrition Sciences Dept.	05/31/2013 Date

Updated 10/31/08

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: July 27, 2012

TO: DAVID B ALLISON, PhD
RPHB-140J 0022
FAX: (205) 975-2540

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Body Composition, Energetic, and Longevity
Sponsor: NIH
Animal Project Number: 120708909

As of July 27, 2012, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	250
Mice	B	300

Animal use must be renewed by July 26, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120708909 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee
CH19 Suite 403
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