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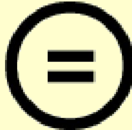

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MASTER'S THESIS

Effects of Laminarin on Gut Microbiota of High Fat Diet-induced Obese Mice

Robin Becina Guevarra

Department of Biotechnology

GRADUATE SCHOOL

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A thesis submitted in partial fulfillment of the requirement

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ABBREVIATIONS

ANOVA	Analysis of variance
AMOVA	Analysis of molecular variance
CTL	Control diet
DNA	Deoxyribonucleic acid
HFD	High-fat diet
HFL	High-fat diet with laminarin
HPLC	High performance liquid chromatography
LEFSE	Linear discriminant analysis effect size
NDS	Non-digestible carbohydrates
NGS	Next generation sequencing
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PEAR	Paired-end read merger
RDP	Ribosomal database project
RT	Retention time
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acid

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ABSTRACT

Edible brown algae are one of the major food materials in Asian countries, especially in South Korea and Japan. In this study, the effects of laminarin on weight gain, fecal short chain fatty acid (SCFA) and gut microbiota of high fat-fed mice were investigated. Mice were fed with 45% Kcal fat-diet (HF), 45% Kcal fat diet supplemented with 1% laminarin (HFL) and a normal diet (CTL) for four weeks. Additional two weeks of feeding trial without laminarin were allowed to investigate the post effects of laminarin. To indirectly measure prebiotics effects, concentrations of fecal SCFA were quantified using High-Performance Liquid Chromatography (HPLC). Fecal microbiota was also analyzed through sequencing V4 region of the 16S rRNA gene by Illumina's MiSeq.

HFL showed no weight increase during the 4th week. Results from taxonomic composition comparison analysis showed that HFL had significantly higher abundance of the phylum *Bacteroidetes* ($P<0.05$) than those of HF but dramatically decreased after the termination of laminarin injection, suggesting that laminarin shifted gut microbiota to what is referred as a 'lean type of microbiota' and the effect did not last more than a week. In addition, HFD and HFL showed significantly lower concentrations of acetic, butyric, formic, lactic, propionic, and pyruvic acid ($P<0.05$) in feces, while isobutyric acid was not detected in any sample. As previously reported, HF and CTL displayed significantly different microbial

communities in response to diet-induced obesity ($P<0.05$). Results from the NMDS analysis positioned HFL microbiota in between CTL and HF. Our results suggested that laminarin could shift gut microbiota toward ‘lean type’ but did not enhance SCFA production in gut. Further studies are required for investigation of prebiotics effects of laminarin.

INTRODUCTION

Obesity and its associated disorders has increased substantially worldwide over the last decades thus, it requires proper treatment; people suffering from it typically consume a larger amount of food which leads to changes in intestinal microbiome (Shcherbakova et al., 2015). Recent insight suggests that an altered composition and diversity of gut microbiota could play an important role in the development of metabolic disorders such as obesity (Moreno-Indias et al., 2014). Currently, there are many strategies in order to restore or modulate the gut microbiota composition for the treatment and prevention of inflammatory diseases including obesity (Erejuwa et al., 2014; West et al., 2015). For example, targeting the microbiota using antibiotics, probiotics, prebiotics or even fecal transplants are considered as promising strategies for the treatment of obesity (Villanueva-Millan et al., 2015).

In recent years, the use of potential natural products to combat obesity has increasingly become of considerable interest (Torres-Fuentes et al., 2015). Modulating the gut microbiota with non-digestible carbohydrates (NDC) may exert anti-obesogenic effects (Hobden et al., 2015) such that higher intakes of fiber are linked to lower body weights (Slavin, 2013). These oligosaccharides are best known as “prebiotics”, “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-bring and health” (Gibson et al., 2004; Gibson and

Roberfroid, 1995). However, much of the researches to date have focused on well-established prebiotics such as inulin and fructo-oligosaccharides (Hobden et al., 2015; Morris and Morris, 2012; Rastall, 2010).

Currently, significant attention is paid to the use of polysaccharides from marine algae as potential prebiotics (Zaporozhets et al., 2014). Edible brown algae are traditionally consumed in Asia, particularly in Republic of Korea, Japan and China (An; Kuda; et al., 2013). Brown algae contain water-soluble polysaccharides that are regarded as dietary fibers, such as alginate, fucoidan and laminarin (An; Yazaki; et al., 2013; O'Sullivan et al., 2010). Furthermore, brown algal polysaccharides are not digested by intestinal enzymes; instead they are fermented by the intestinal/fecal microbiota in humans and rats (An; Yazaki; et al., 2013; Kuda et al., 2009). Alginate and fucoidan are located in the cell wall matrix while laminarin is a -1, 3-glucan contained in cells as a storage polysaccharide (Kuda et al., 2002). Beta-glucans (-glucan) have been shown to exhibit prebiotic properties by increasing the populations of bifidobacteria and lactobacilli spp. in the large intestine. However, the functions of polysaccharides from marine macroalgae as potential prebiotics for both humans and animals are still not wholly understood.

In this study, we investigated whether laminarin exhibits biological properties that may benefit the host's health to determine its value as a potential prebiotic. It is hypothesized that the inclusion *Laminaria digitata* seaweed extracts on high-fat-induced obese mice will reduce its weight gain and microbial community shift will be observed. The objective of this experiment was to evaluate the effects of

dietary supplementation with laminarin, a polysaccharide extract from *L. digitata*, on weight gain, fecal short-chain fatty acid concentrations and gut microbiota of high-fat fed mice.

MATERIALD AND METHODS

Animals and diet

This study was conducted in conformity with the policies and procedures by Laboratory Animal Center, Jeju National University (Republic of Korea). Twenty-seven female BALB/c 4 week-old mice (Orient Bio Inc., Republic of Korea) were housed in stainless wire cages in a room maintained at 20–24 ^{o}C with 55±5% relative humidity and controlled lighting (12 h light/dark cycle). Animals were randomly assigned to three dietary groups (n = 3/group) and housed in groups of three mice per cage. After 1 week of acclimatization, the mice were fed standard diet (CTL), diet containing 45%Kcal fat diet (HFD) and HFD containing 1% laminarin (HFL) for four weeks. Additional two weeks of feeding trial without laminarin were allowed to investigate the post effects of laminarin. Both solid diet and water were consumed *ad libitum*. At the end of the experiment, the mice were bled from the abdominal aorta under diethyl ether anesthesia. The cecum was removed and then stored at -20°C prior to downstream analysis. Fecal sampling and body weight was recorded weekly. Growth rate was calculated as the difference between the final and initial weights divided by the 42 days intervention period.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from fecal powders using the MOBIO Power Fecal DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). The V4 region of the

16S rRNA gene was amplified by polymerase chain reaction (PCR), as previously described (Kozich et al. 2013). Two microliters of total DNA from each sample was used as a template. The DNA was amplified in triplicate using the Maxime PCR PreMix Kit (iNtRON Biotechnology Inc., Gyonggi, Korea) using the following reaction conditions: 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 55 °C for 15 s, and 72 °C for 1 min, and 72 °C for 5 min. The obtained PCR products were further purified using Exo-AP PCR Clean-Up Mix (Doctor Protein Inc., Seoul, Korea). All DNA samples were quantified using Qubit (Invitrogen, Carlsbad, CA, USA). The equimolar purified amplicons were pooled and stored at −20 °C until sequencing. The Illumina MiSeq platform (Illumina, San Diego, CA, USA) was used to sequence the V4 region of the 16S rRNA gene. Construction of the gene library and sequencing were conducted at Macrogen Inc. (Seoul, Korea), according to the manufacturer's instructions.

Sequence processing and analysis

The sequences generated from MiSeq sequencing were mainly analyzed with the free software Mothur for identification of operational taxonomic unit (OTU), taxonomic assignment, bacterial community comparison and statistical analysis. First, paired-end reads obtained from a MiSeq run were assembled using the 'PEAR' software (Zhang et al. 2014). Index sequences were trimmed, subsequently aligned to similar sequences in the SILVA rRNA database (Quast et al. 2013), screened, and finally filtered using the Mothur pipeline (Schloss et al. 2009). Artificial erroneous

reads were corrected using the pre.cluster Mothur subroutine, and chimeric sequences were removed using UCHIME (Edgar et al. 2011). Taxonomic classification was done using the Ribosomal Database Project (Cole et al. 2009) training set version 10, followed by non-archaeal/bacterial sequence removal based on the taxonomic classification results. Prior to cluster analysis, all singleton sequences were removed, as suggested previously (Degnan and Ochman 2012). Operational taxonomic units (OTUs) were calculated at a distance of 0.03, using the Mothur subroutine cluster.split, and OTU-consensus taxa were determined using the classify.otu Mothur subroutine. Representatives of resultant OTUs were taxonomically identified using the SILVA taxonomy tool. Dissimilarities in the bacterial community were analyzed based on the Yue and Clayton theta coefficient, calculated using tree.shared, and visualized in a tree format.

Bacterial community comparison analysis

To examine the temporal stability of the microbial community among the three treatment groups, sequencing reads from each sample were assigned as an OTU with 97% sequence identity. The OTU information from each sample was then transferred into a dendrogram with the tree.shared script of MOTHUR. A newick-formatted tree file was generated through this analysis. The distances between microbial communities from each sample were calculated using Yue and Clayton theta coefficient. The parsimony command was used to determine whether the clustering within the tree is statistically significant. The distance matrix generated by dist.shared command of MOTHUR was visualized using non metric multi-

dimensional scaling (NMDS) plot. NMDS was performed to represent the multidimensional relationships among the samples in a low dimensional space. Moreover, two dimensions were used with NMDS command of MOTHUR to preserve the distance between the samples. Analysis of molecular variance (AMOVA) was used to statistically test the spatial separation in the NMDS plot among the three treatment groups. Correlation of the relative abundance of each OTU with the two axes in the NMDS plot was determined using corr.axes command of MOTHUR. This was done to determine which OTUs are responsible for the shifting the samples along the two axes. Lines radiating from the origin were plotted using 'Arrows' graphical command in R software. Metastats command in MOTHUR was also used to differentiate the different groupings of samples. It is a non-parametric t-test that determines whether there are any OTUs that are differentially represented among the three treatment groups in this study.

Calculation of species richness and diversity indices

Shannon's diversity, inverse Simpson, Chao I richness indices and rarefaction curves were calculated using the MOTHUR program. The 3% dissimilarity cutoff value was used for assigning an OTU. Good's coverage was calculated as $G = 1 - n/N$, where n is the number of singleton and N is the total number of sequences in the sample.

Analysis of shared OTUs

Shared OTUs were determined based on the common OTUs among the three experimental groups. The Cytoscape software (Shannon et al., 2003) was used to draw a network map of common OTUs and preferred layout was applied.

Preparation of fecal samples for SCFA analysis

The SCFAs of faecal samples were extracted as described by (Huda-Faujan et al., 2010) method. Faecal samples of weight 0.2 g were used and diluted at ratio 1:4 to 1:8 (w/v) in HPLC grade water. The samples were then vortexed for 1 min and the homogenate was centrifuged at 10,000g for 10 min. The SCFA-containing supernatant was filtered through cellulose acetate membrane with a pore size of 0.2 μm (Advantec MFS, Inc., Japan) and stored at -20°C until HPLC analysis.

Chemicals and Reagents

Seven commercial organic acids were used in this study. Formic acid, 98%, pyruvic acid and n-Butyric acid, 99% were obtained from Kanto Chemical Co., Inc., Japan. Propionic acid, >99% and lactic acid, 100% were obtained from Daejung Chemicals and Metals Co., Republic of Korea. Acetic acid, >99% were purchased from Sigma-Aldrich Co., Republic of Korea. Isobutyric acid, 99% was purchased from Junsei Chemical Co., Japan. HPLC grade water and acetonitrile was obtained from Fisher Scientific, UK.

Determination of Short-Chain Fatty Acids

SCFAs analyses were carried out by using HPLC. Briefly, 40 μl of fecal extracts were injected directly into HPLC System (Shimadzu LC-10AD Liquid Chromatography) with Shimadzu SPD-M20A Prominence HPLC Photo Diode Array Detector (Shimadzu, Kyoto, Japan). SCFA in fecal samples were separated using an ionic exchange resin, Aminex HPX-87H column, (Aminex HPX-87H, 300 x 7.8 mm, Bio-Rad Laboratories, Richmond, USA) at 65°C. The target compounds were detected using a UV detector set at wavelength of 210 nm. Filtered 0.01 N H2SO4, through 0.45 μm nylon membrane (Sigma-Aldrich, Republic of Korea), was used as a mobile phase at a flow rate of 0.6 ml/ min.

Preparation of Calibration Standard Curve

Quantification of SCFAs in fecal samples was carried out using external calibration standard curves method. Seven calibration standards were prepared at six levels of concentration ranging from 0.005M to 0.03M for pyruvic acid, 0.01M to 0.06M for formic acid and acetic acid, and 0.02M to 0.12M for lactic acid, propionic acid, isobutyric acid and butyric acid. The reference samples were injected repeatedly for at three times to measure the retention time. The calibration curves were constructed by plotting the relative peak area versus the molarity of solution. Fecal SCFA concentrations were expressed as mean μmol per gram wet weight feces.

Statistical analysis

Significant differences between microbial communities were examined based on a p-value obtained using analysis of molecular variance (AMOVA) and Tukey's multiple comparison test. Metastats analysis was also used to conduct differential abundance tests between read-abundance and treatments.

RESULTS AND DISCUSSION

Effect of laminarin on weight gain of high fat diet-fed mice

The changes in body weight during the course of the six-week experiment were expressed relatively (Figure 1). HFD hastened the increase in body weight gain during the course of the 6-week experiment as compared to control (P<0.001). This result is in agreement with previous studies on high fat diet–fed mouse model (Neyrinck et al., 2008; Winzell and Ahren, 2004). HFD and HFL fed mice were significantly different from the CTL group (P<0.001). Four weeks after feeding trial, inclusion of laminarin in HFL group was terminated to observe the post-effects of laminarin. It was observed that laminarin seemed to lose its effects on body weight gain of high fat-fed mice after termination of laminarin feeding. At week 4, laminarin significantly suppressed the effect of high fat diet on body weight gain (P<0.01). In Figure 2, the relative weight gain of HLF group at week 4 was significantly different from HFD but was not significantly different from CTL (P<0.01). However, HFL group started to gain weight again after week 4.

Several studies suggest that dietary fibers could play a role in the control of obesity and associated metabolic disorders (Delzenne and Cani, 2005; Galisteo et al., 2008; Neyrinck et al., 2008). Recent data suggest that the gut microbiota can be related to obesity and metabolic response towards a HFD (Backhed et al., 2007; Cani et al., 2008; Turnbaugh et al., 2006). The aim of this study was to investigate whether

laminarin exhibits biological properties that may benefit the host's health to determine its value as a potential prebiotic. Addition of laminarin at a concentration of 1%, representing only few percentages of fermentable fiber, is probably not sufficient to induce important gut fermentation. Therefore, it should be interesting to increase the concentration of laminarin in the diet to further investigate its effects in animal models of systemic inflammatory diseases. Here, it has shown that the modulation of weight gain by laminarin occurs as early as 4 weeks after the beginning of the dietary treatment. However, it is interesting to know that body weight increases again upon termination of laminarin feeding and it does not necessarily lead to an improvement of gut microbiota associated to obesity.

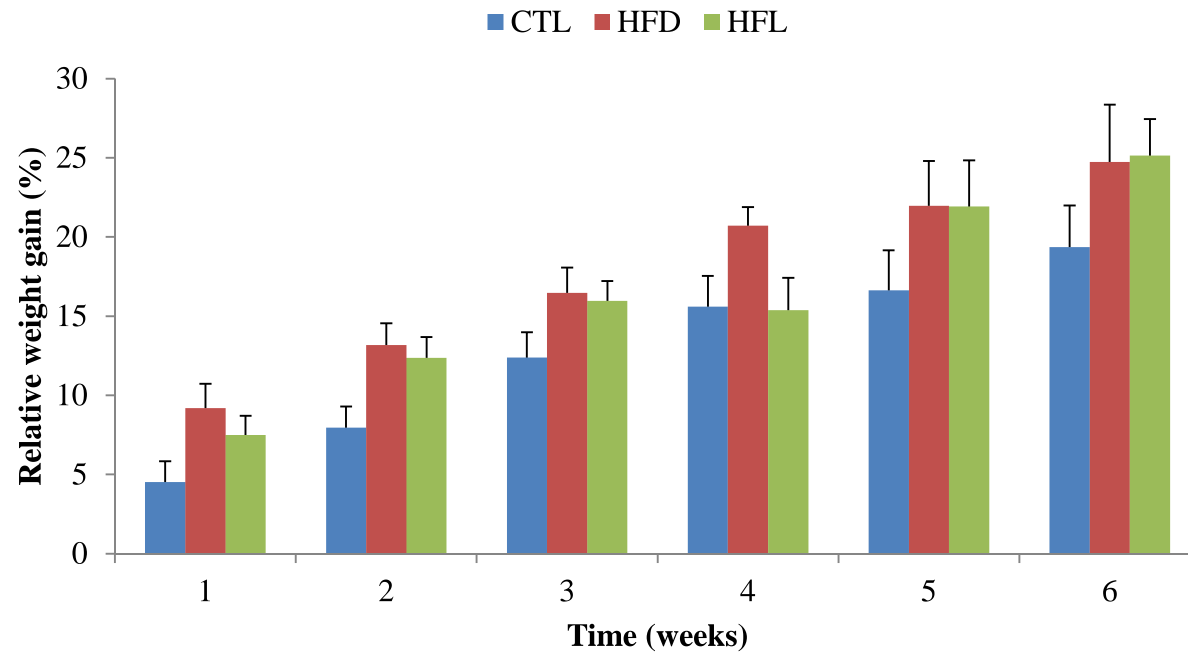

Figure1. Relative weight gain of CTL, HFD and HFL fed mice group. The first four weeks were laminarin feeding trial and additional two weeks without laminarin in HFL group. Each bar represents the mean of three measurements and error bars show standard error.

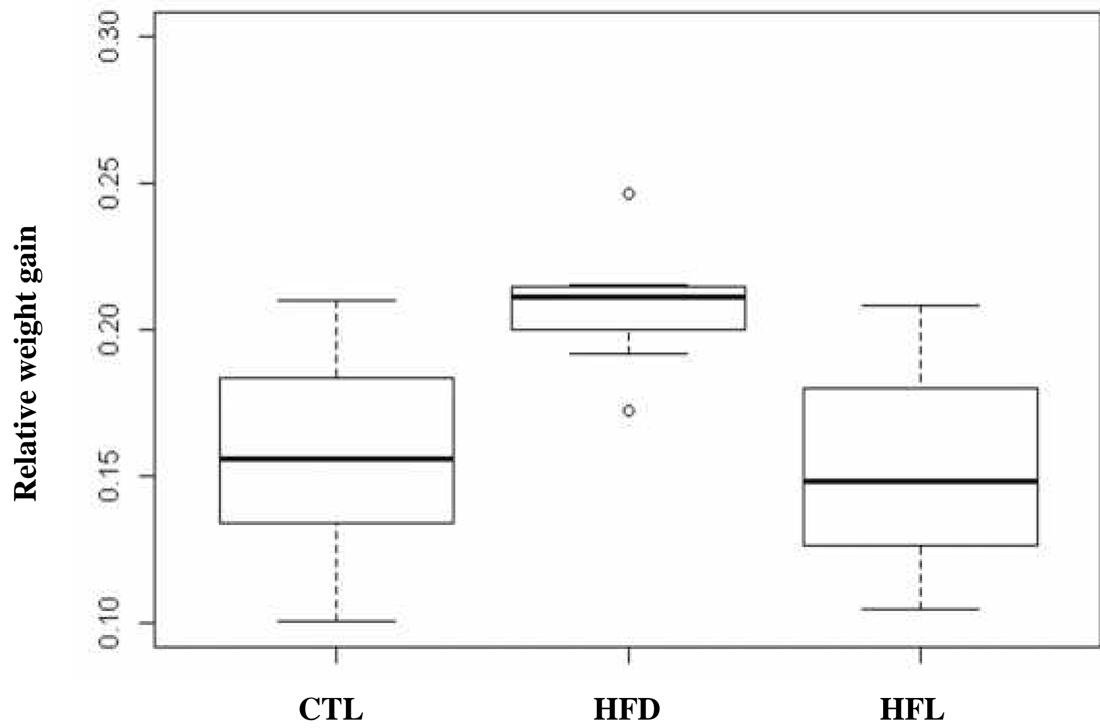

Figure2. Box-and-whiskers plot of relative weight gain of CTL, HFD and HFL fed mice at week 4.

Sequencing depth and alpha diversities

To assess the impact of laminarin feeding on bacterial communities of high fat fed obese mice, we sequenced V4 amplicon of 16S rRNA gene. A total of 63 fecal samples were collected from CTL (n = 9), HF (n=9) and HFL (n = 9) mice group in a 42 day feeding trial. DNA was extracted from these samples and was subjected to sequencing of the V4 region of the 16S rRNA gene. Sequences were processed and analyzed using Mothur v1.35 (Schloss et al., 2009). Sequencing error and chimeras were detected and removed by using the default settings in Mothur. In this study, we applied the curation pipeline developed by Kozich et al. (Kozich et al., 2013) and also added split.abund Mothur subroutine after pre.cluster subroutine to remove singletons. A total of 2,589,983 high quality reads were obtained from 63 fecal samples. Resulting number of reads per sample ranged from 7144 to 134,859 with high Good's coverage (>0.99). These sequences were assigned to 1886 operational taxonomic units (OTUs) based on 97% similarity. The summary of the alpha diversity indices in CTL, HFD and HFL fed mice group is presented in Table 1, 2, and 3, respectively.

For the downstream alpha and beta diversity analyses, the number of sequences was normalized to 7144 using "sub.sample" command in Mothur to normalize the number of sequence reads for each sample. The subsampled sequences still yielded enough bacterial communities as shown in Good's coverage and rarefaction analysis (Figure 3).

Table 1. Number of sequences analyzed, sample coverage, number of OTUs, observed OTU richness, (Chao1), and OTU evenness (Inverse Simpson) for 16S rRNA libraries of CTL mice fecal samples.

week	group	# of sequence	coverage	# of OTUs	Inverse Simpson	Chao1
D0	CTL	7144	0.99	264	4.47	352.32
D0	CTL	7144	0.99	285	7.07	354.16
D0	CTL	7144	0.99	272	8.07	339.08
W1	CTL	7144	0.99	229	4.06	296.20
W1	CTL	7144	0.99	248	5.07	309.29
W1	CTL	7144	0.99	233	2.75	280.63
W2	CTL	7144	0.99	288	18.12	338.83
W2	CTL	7144	0.99	277	9.75	322.64
W2	CTL	7144	0.99	251	8.45	300.70
W3	CTL	7144	0.99	291	21.90	352.12
W3	CTL	7144	0.99	306	26.76	387.08
W3	CTL	7144	0.99	283	16.23	332.91
W4	CTL	7144	0.99	285	16.84	366.00
W4	CTL	7144	0.99	272	9.36	341.84
W4	CTL	7144	0.99	303	17.01	357.02
W5	CTL	7144	0.99	286	17.66	346.02
W5	CTL	7144	0.99	289	17.57	404.18
W5	CTL	7144	0.99	270	21.54	310.32
W6	CTL	7144	0.99	246	7.47	339.95
W6	CTL	7144	0.99	289	16.03	404.62
W6	CTL	7144	0.99	327	31.24	428.48

Table 2. Number of sequences analyzed, sample coverage, number of OTUs, observed OTU richness, (Chao1), and OTU evenness (Inverse Simpson) for 16S rRNA libraries of HFD mice fecal samples.

week	group	# of sequence	coverage	# of OTUs	Inverse Simpson	Chao1
D0	HFD	7144	0.98	271	6.15	352.61
D0	HFD	7144	0.99	281	10.38	332.07
D0	HFD	7144	0.99	259	11.87	306.83
W1	HFD	7144	0.99	173	7.64	326.21
W1	HFD	7144	0.99	86	5.34	122.11
W1	HFD	7144	0.99	148	7.74	217
W2	HFD	7144	0.99	191	12.80	231.83
W2	HFD	7144	0.99	144	11.71	187.33
W2	HFD	7144	0.99	143	6.08	168.14
W3	HFD	7144	0.99	178	17.08	233.65
W3	HFD	7144	0.99	138	6.37	163.83
W3	HFD	7144	0.99	180	11.88	264.18
W4	HFD	7144	0.99	199	27.57	242
W4	HFD	7144	0.99	116	4.28	154.15
W4	HFD	7144	0.99	159	11.30	184.59
W5	HFD	7144	0.99	165	13.25	243
W5	HFD	7144	0.99	114	3.86	155.33
W5	HFD	7144	0.99	197	25.70	315.19
W6	HFD	7144	0.99	166	10.35	205.60
W6	HFD	7144	0.99	125	5.91	212.88
W6	HFD	7144	0.99	141	4.73	169.12

Table 3. Number of sequences analyzed, sample coverage, number of OTUs, observed OTU richness, (Chao1), and OTU evenness (Inverse Simpson) for 16S rRNA libraries of HFL mice fecal samples.

week	group	# of sequence	coverage	# of OTUs	Inverse Simpson	Chao1
D0	HFL	7144	0.99	258	11.69	325.162
D0	HFL	7144	0.99	238	3.74	281.33
D0	HFL	7144	0.99	225	2.89	309.09
W1	HFL	7144	0.99	155	8.77	192.06
W1	HFL	7144	0.99	154	14.09	206
W1	HFL	7144	0.99	156	11.09	199.59
W2	HFL	7144	0.99	146	4.79	222.15
W2	HFL	7144	0.99	149	9.55	203.47
W2	HFL	7144	0.99	126	5.62	154.05
W3	HFL	7144	0.99	98	5.08	121.21
W3	HFL	7144	0.99	144	10.41	207.07
W3	HFL	7144	0.99	127	11.02	172.77
W4	HFL	7144	0.99	116	5.62	147.07
W4	HFL	7144	0.99	150	6.59	179.64
W4	HFL	7144	0.99	141	5.88	155.04
W5	HFL	7144	0.99	157	21.18	207.17
W5	HFL	7144	0.99	114	8.05	130.50
W5	HFL	7144	0.99	138	10.72	156.06
W6	HFL	7144	0.99	145	6.54	201.4
W6	HFL	7144	0.99	155	6.25	200.15
W6	HFL	7144	0.99	154	7.89	199

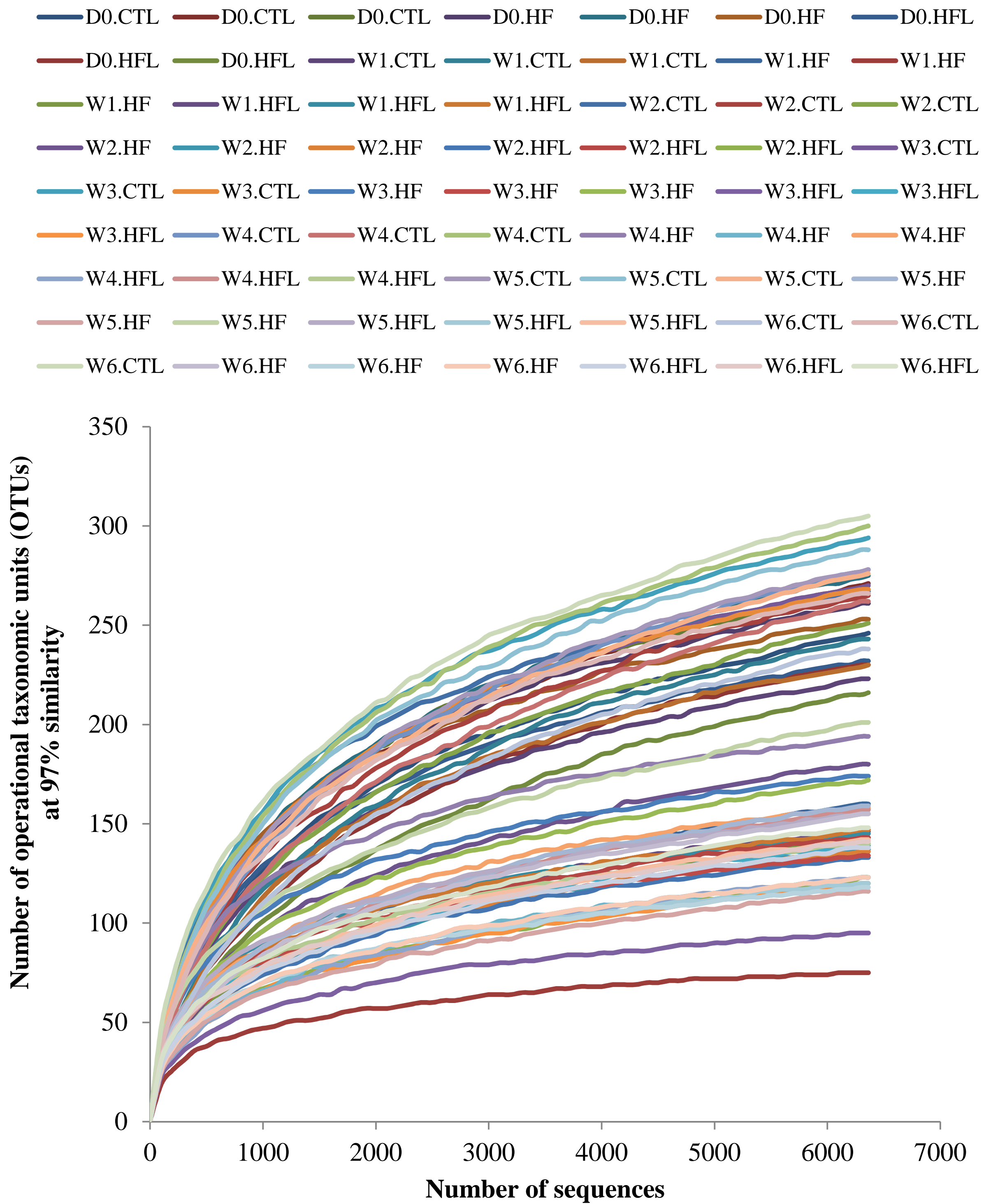

Figure 3. Rarefaction analysis of V4 region of the 16S rRNA gene sequences in fecal microbiota of CTL, HFD, and HFL diet fed mice groups. Rarefaction curves were constructed at a 97% sequence similarity cut-off value by Mothur.

Shannon index and inverse Simpson index was used as a measure of bacterial community diversity among the three mice groups (Figure 4 and 5). Both diversity indices were significantly different among CTL, HF and HFL group ($P<0.05$). The community richness showed that CTL group was significantly higher than HF and HFL group ($P<0.001$). However, the community richness between HFD and HFL was not significantly different.

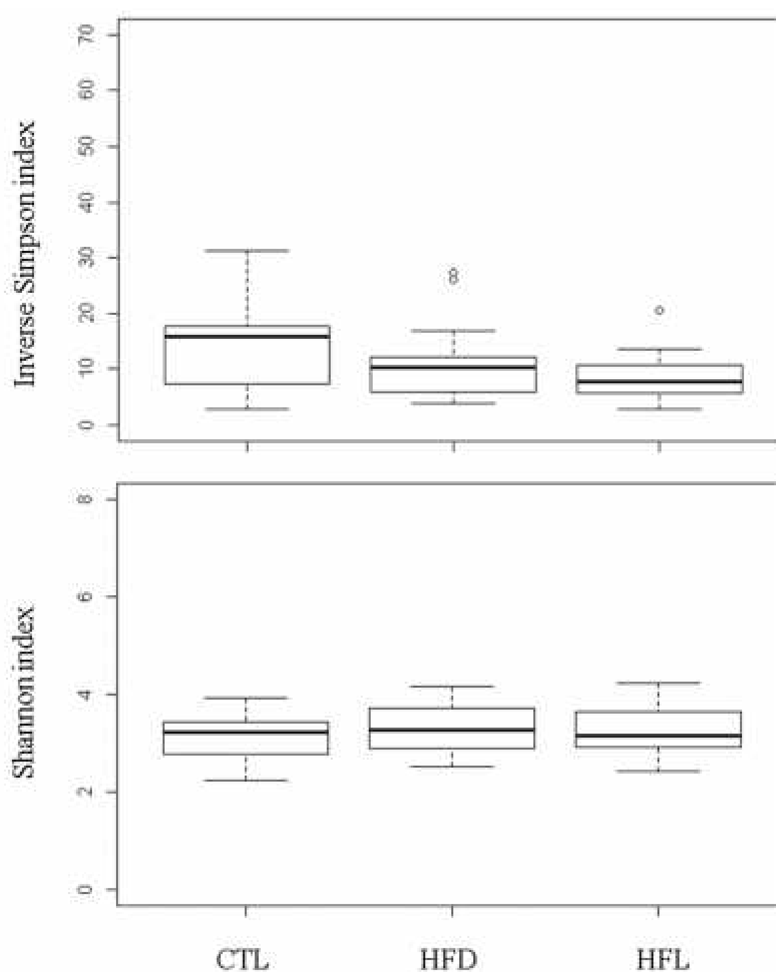

Figure 4. Comparison of bacterial community alpha diversity among the CTL, HF and HFL mice group. Diversity was measured by inverse Simpson and Shannon index.

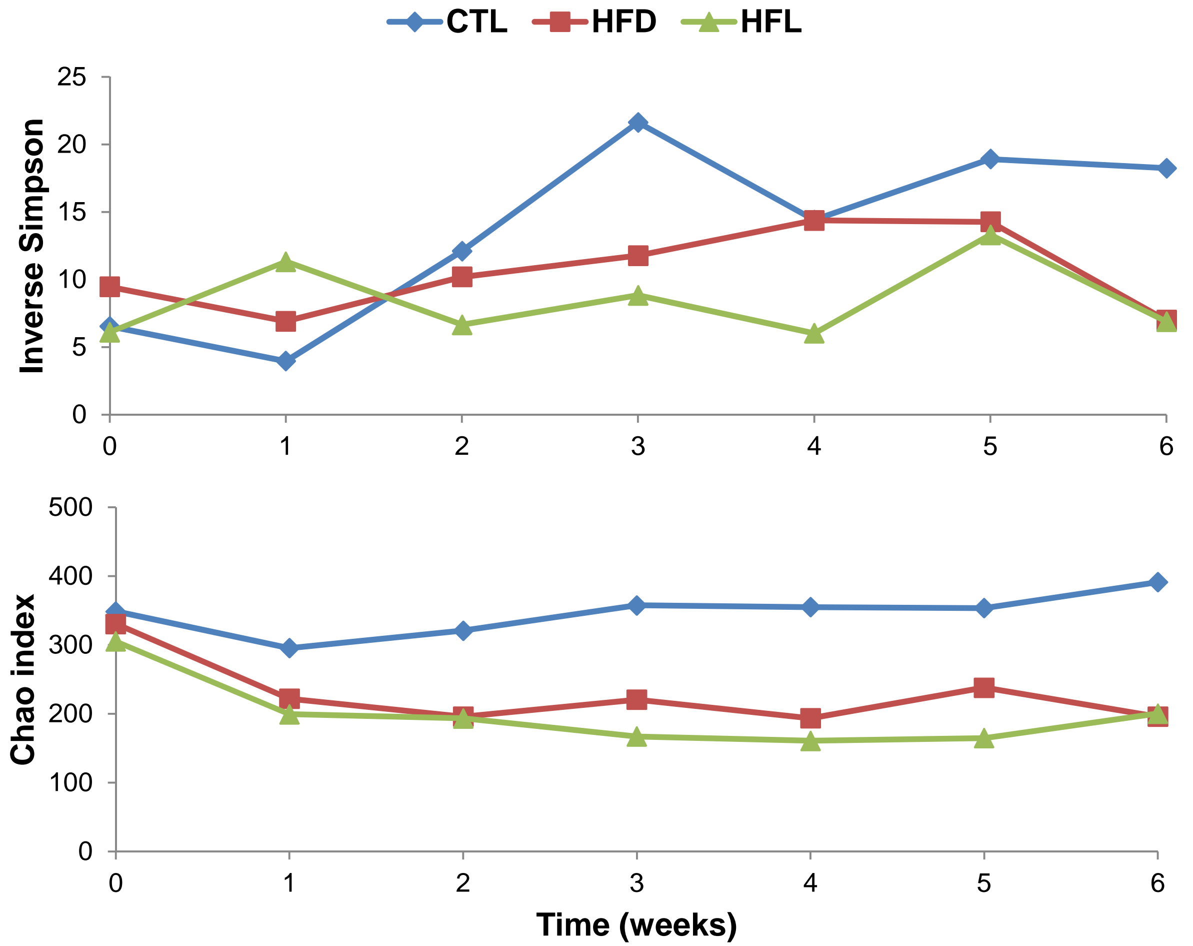

Figure 5. Variation of community alpha diversities among CTL, HFD and HFL fed mice group throughout the six-week feeding trial of laminarin. Diversity was measured by inverse Simpson and Chao index.

Bacterial community membership and structures using OTU-based approach

Clustering analysis. After comparing the alpha diversities among the three treatment groups, beta diversity measures were also examined among the CTL, HFD and HFL fed mice. We determined the relationship between the gut microbiota from the three treatment groups: CTL, HFD and HFL by using Bray-Curtis dissimilarity index (Bray and Curtis, 1957), which were visualized by a dendrogram (Figure 6). Each branch on the tree represents one gut microbiota. Red, blue and green circles indicate gut microbiota from CTL, HFD and HFL fed mice, respectively. Interestingly, there were two major clusters of fecal gut microbiota observed in the dendrogram. CTL fed mice were clustered into one cluster and gut microbiota from HFL were closer to those of the HFD fed mice. CTL gut microbiota at day 0 can be found at the HFD and HFL branches since the gut microbiota of the three treatment groups were assumed to be equal at the beginning of the study.

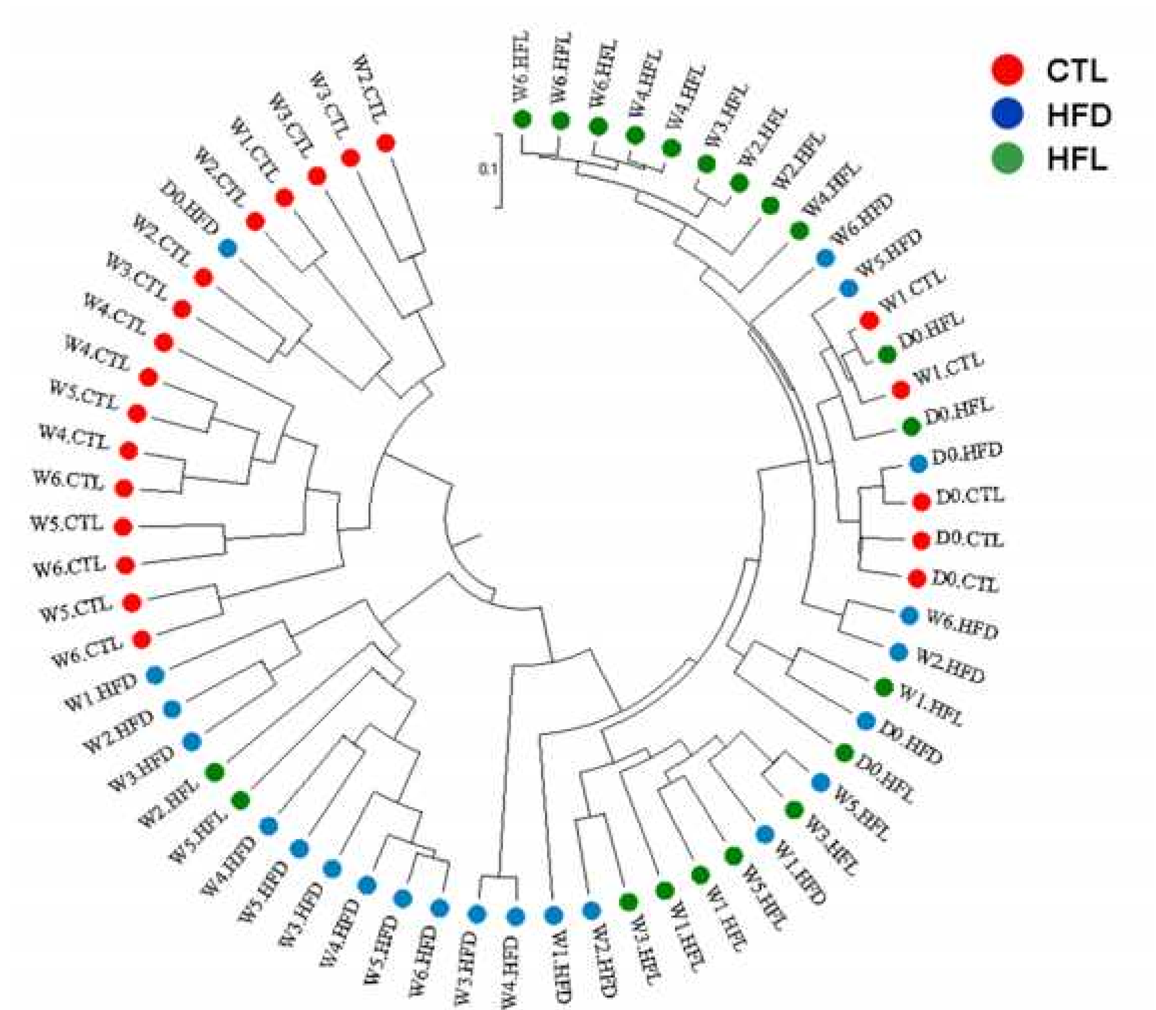

Figure 6. Clustering analysis of the gut microbiota of CTL, HFD and HFL fed mice. Gut microbiota tree was generated based on the Bray-Curtis distances generated by Mothur. The vertical scale bar indicates 10% sequence divergence.

NMDS biplot analysis. Two-dimensional non-metric multidimensional scaling (NMDS) was performed to represent the multidimensional relationships among the samples in a low dimensional space (Figure 7). To estimate the dissimilarities in community membership, Yue and Clayton (Theta YC) distances (Yue and Clayton, 2005) was calculated, which takes into account both membership and relative abundance. Red squares, blue triangles and green circles represent the gut microbiotas from the CTL, HFD and HFL fed mice, respectively. Distances between symbols on the ordination plot reflect relative dissimilarities in community structures. Consistent with the dendrogram, clustering patterns appeared to be similar. On the NMDS plot, each symbol represents one gut microbiota, where a shorter distance between points indicates increasing similarity. NMDS plot showed that there were three groupings based on the proximity of the objects belonging to each group. The gut microbiotas were clustered according to diet. The gut microbiota of the CTL fed mice was distinct from those of the HFD and HFL fed mice groups. Furthermore, the bacterial communities of HFL were clustered in between the CTL and HFD. Interestingly, analysis of molecular variance (AMOVA) of the Bray-Curtis distances showed that the differences among the three treatment groups were statistically highly significant (AMOVA, $P<0.001$) (Table 4). This result is consistent with the alpha diversity, where CTL fed mice group were found to have significantly higher number of OTUs than the HFD and HFL.

To determine which OTUs were responsible for shifting the gut microbiota along the two axes, a biplot was generated using the NMDS axes from the Yue and Clayton (Theta YC) distances. The length of the line connecting the taxon to the

center of the plot is equivalent to the weight of the taxon profile in the final solution of the samples (i.e., the longer the line, the larger part of the inertia it explains). The direction of the lines indicates the sample orientation of its weight. The biplot arrows show the 10 most significant consensus taxonomies contributing to variation along the two axes ($P<0.05$). These taxa were computed based on Spearman correlation of the relative abundance of each OTU. Among the OTUs, two OTUs from the phylum *Bacteroidetes* were most strongly correlated with lean type microbiota (CTL group). Seven OTUs affiliated with phylum *Firmicutes* were correlated with obese type of microbiota pointing to HFD and HFL fed mice. These results indicate that the relative abundance of specific OTUs in the community structures could be associated with the diet.

Table 4. Analysis of Molecular Variance and Bray-Curtis dissimilarity index among the CTL, HFD and HFL fed mice before and after laminarin feeding.

Comparison	Bray-Curtis index	AMOVA p-value (Before)	AMOVA p-value (After)
HFL vs CTL	0.53	<0.001	<0.001
HFL vs HFD	0.32	0.002	0.203
CTL vs HFD	0.53	<0.001	0.002

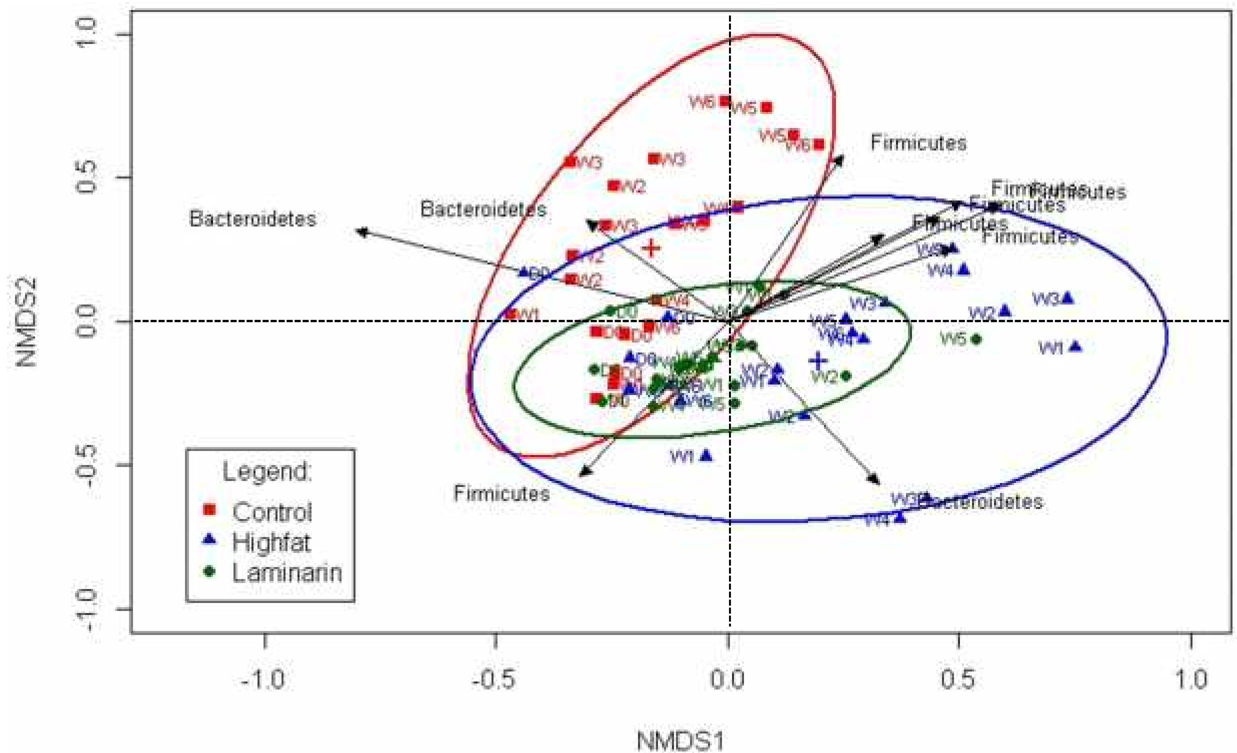

Figure 7. Two-dimensional non-metric multidimensional scaling (NMDS) biplot analysis of the gut bacterial communities of the three experimental treatment groups (CTL, HFD and HFL) based on Bray-Curtis dissimilarity index distances. Red squares, blue triangles and green circles represent the gut microbiotas from the CTL, HFD and HFL fed mice, respectively. Distances between symbols on the ordination plot reflect relative dissimilarities in community structures. The length of the line connecting the taxon to the center of the plot is equivalent to the weight of this taxon profile in the final solution of the samples (i.e., the longer the line, the larger part of the inertia it explains). The direction of the lines indicates the sample orientation of its weight.

Taxonomy-based microbial community analysis

The distribution of sequences at the phylum and genus level in CTL, HFD and HFL fed mice during the six-week intervention is illustrated in Figure 8. All the sequences were found to be associated with 10 bacterial phyla namely *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, *Proteobacteria*, *Deferribacteres*, *Tenericutes*, *Planctomycetes*, *Armatimonadetes* and *Candidatus_Saccharibacteria*. The phylum *Firmicutes* and *Bacteroidetes* dominated the bacterial communities of all the diet groups and together gathered 94.6% (SD = 7.9%) of the sequences. These results were in agreement with previous studies attributing that majority of the human gut microbiota consists of these two major phyla (Nam et al., 2011; Turnbaugh and Gordon, 2009; Turnbaugh et al., 2009). We also examined the relationship between gut microbial community and age of animals in the three diet groups as previous studies have reported a higher *Firmicutes* to *Bacteroidetes* (F/B ratio) in obese individuals than lean individuals (Ley et al., 2005). This study revealed that there was a strong positive linear relationship between F/B ratio and age of HFD fed mice ($R^2 = 0.54$), however no relationship was found between F/B ratio and age in CTL ($R^2 = 0.04$) and HFL ($R^2 = 0.003$) fed mice.

Figure 8B shows the relative abundance of the sequences at genus level. All of the sequences were associated with 80 bacterial genera. *Bacteroides* dominated in all diet groups (CTL, 23.4%; HFD, 13.9%; and HFL, 36.3%), while more than half the sequences were unclassified. The unclassified genera could be simply owing to

the short read length (250 bp) or existence of unculturable bacteria in feces that are not in the Ribosomal Database Project (RDP) database (Kim et al., 2015).

Core gut microbiota of the three dietary groups

A major interest in research on gut communities is to determine whether core microbiota exist that are broadly distributed among individuals. To identify specific genera that were differentially abundant in each diet groups, Metastats (Paulson et al., 2011) and linear discriminant analysis effect size (LEfSe)(Segata et al., 2011) were performed. These are robust tools that focus not only on statistical significance but also biological relevance. Statistically abundant genera ($P<0.05$) were represented in a heatmap with a dendrogram added (Figure 9). A total of 243 OTUs were significantly differentially represented among the three treatment groups, with 154, 48 and 41 more abundant OTUs in CTL, HFD and HFL fed mice group, respectively. Heatmap analysis showed that the genera *Lactococcus*, *Akkermansia*, *Oscillibacter*, *Clostridium_XI*, *Clostridium_IV* and *Clostridium_XIVb* were the predominant members in the gut microbiota of HFD fed group. Moreover, taxa belonging to the genus *Bacteroides*, *Clostridium_XVIII*, *Clostridium_XlVa*, *Parabacteroides*, *Bifidobacterium*, *Asaccharobacter*, and *Streptococcus* were significantly increased by laminarin feeding in high fat diet-fed mice. In the CTL group *Allistipes*, *Clostridium_XlVa*, *Lactobacillus*, *Clostridium_XlVb*, *Butyricicoccus*, *Oscillibacter*, *Anaeroplasma*, and *Acetitomaculum* were significantly abundant in CTL fed mice group.

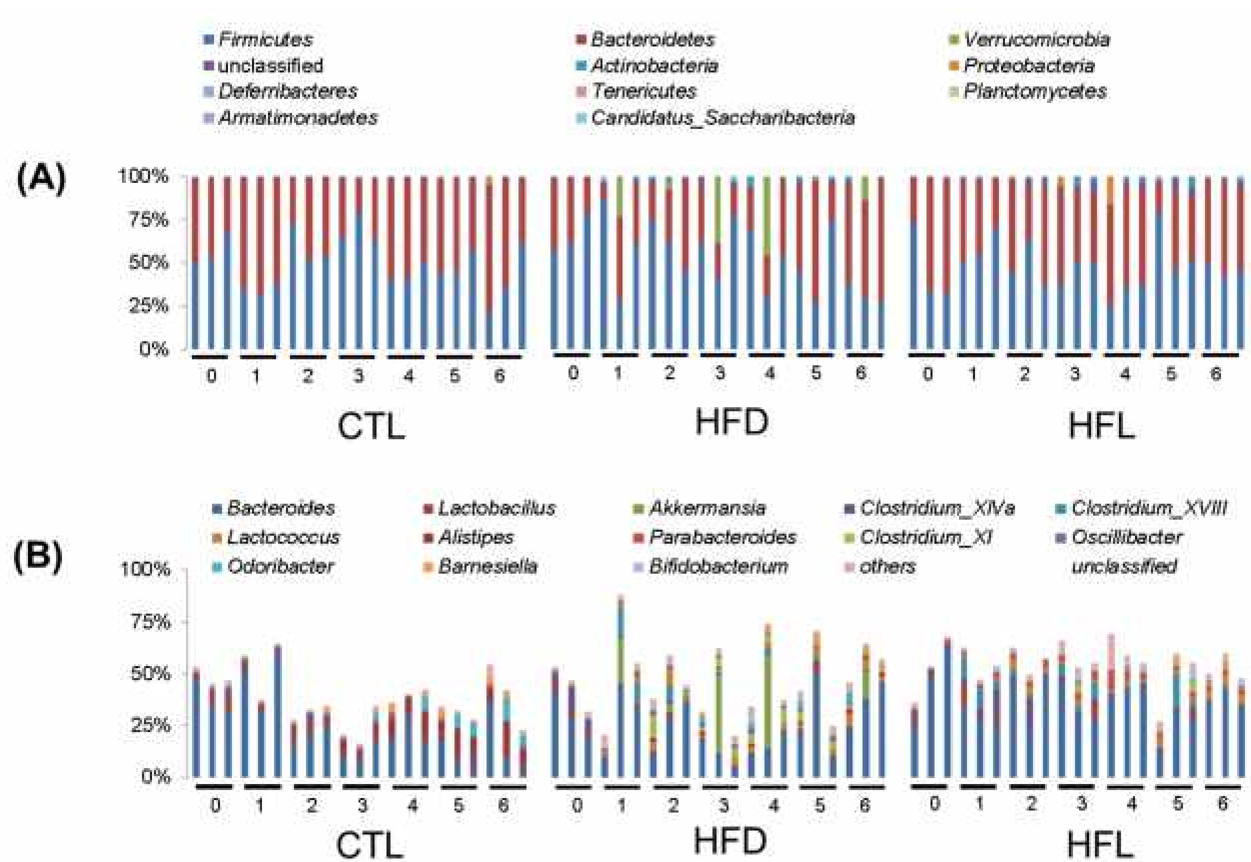

Figure 8. Relative abundance of the sequences at the phylum (A) and genus level (B) in the gut microbiota from the CTL, HFD, and HFL fed mice. Each bar in the stacked bar charts represents the relative abundance of individual taxa.

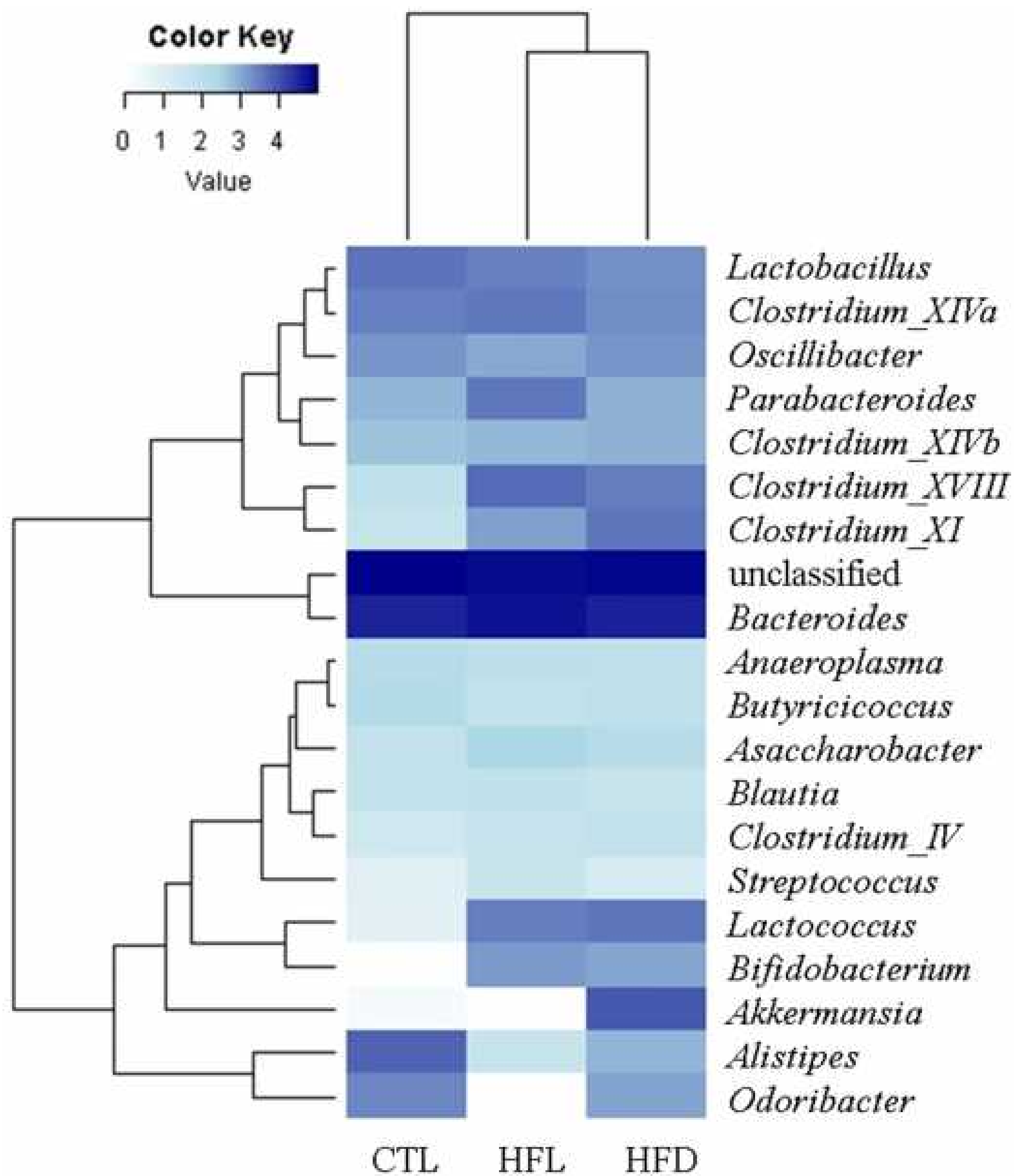

Figure 9. Differentially abundant OTUs among CTL, HFD and HFL fed mice groups identified by metastats (P<0.05). The heat map was created using genus based results after normalization. Dark blue indicates abundant genera and light blue indicates less abundant genera. Each column represents groups and each row indicates genus.

Firmicutes to Bacteroidetes ratio

The ratio of relative abundance of *Bacteroidetes* and *Firmicutes* was previously suggested to differentiate lean and obesity types in humans (Ley et al., 2005; Ley et al., 2006). Figure 10 shows the F/B ratio during the six-week dietary treatment. In the present study, F/B ratio was significantly different at week 4 with HFL group have significantly lower F/B ratio than HFD but not significantly different between CTL fed mice. The reduced proportions of the phylum *Firmicutes* and the increased proportions of phylum *Bacteroidetes* in HFL fed mice compared to HFD group could explain the weight gain loss at week 4. This relationship between the two main phyla has been addressed in a number of studies associated with obesity (Ferrer et al., 2013; Furet et al., 2010; Ley et al., 2006; Turnbaugh et al., 2009). However, series of studies have failed to confirm the study of the original publication by Ley and colleagues (Ley et al., 2006) and have shown inconsistent results with reference to the changes in microbial community structures of obese humans (Santacruz et al., 2010; Santacruz et al., 2009; Schwiertz et al., 2010; Zhang et al., 2009). Yet evidence identifying which specific microbes contribute to or predict obesity is not completely consistent across studies. Further study is required to establish which specific microbial taxa could predict body weight regulation.

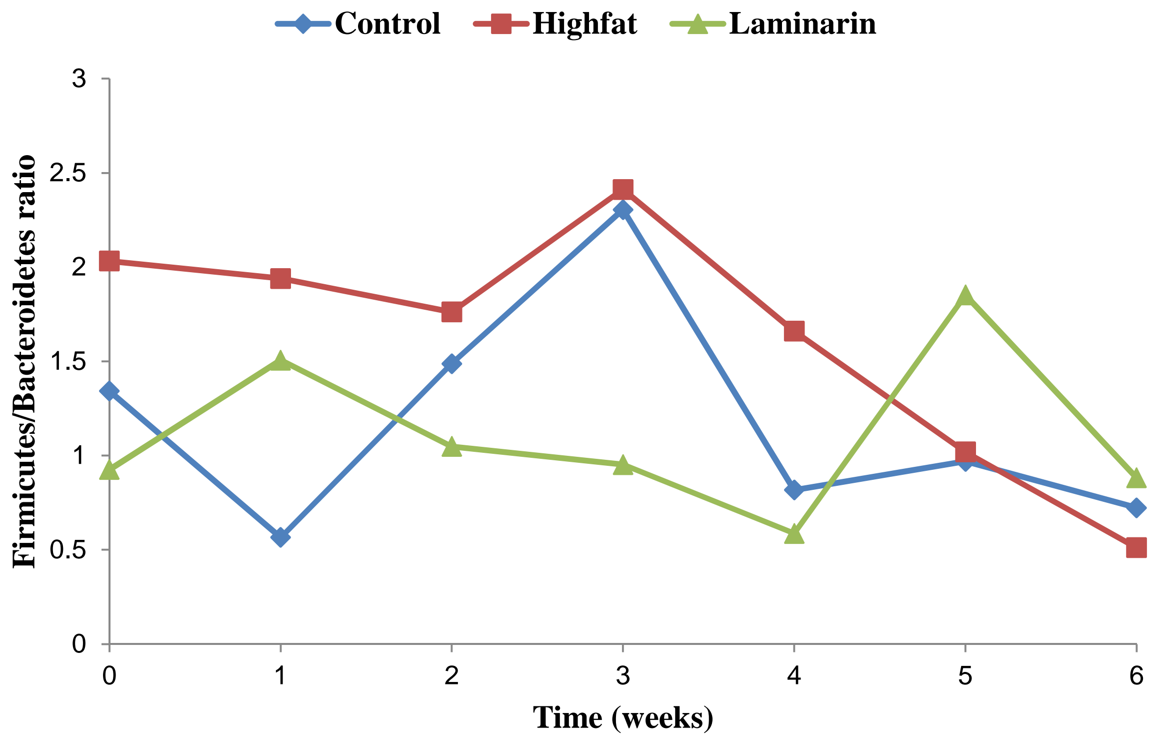

Figure 10. ***Firmicutes*** **to** ***Bacteroidetes*** **ratio during the six-week feeding trial among the CTL, HFD and HFL fed mice group.**

Analysis of shared OTUs

Figure 11 depicts a network-based analysis obtained using Cytoscape program (Ley et al., 2008; Shannon et al., 2003) of fecal microbiota before and after laminarin feeding. Red, blue and green circles denote CTL, HFD and HFL fed mice group, respectively. Small white dots correspond to OTUs and each line indicates that an OTU was identified from the same diet group. This analysis revealed that during laminarin feeding, microbial communities from the three treatment groups were highly significantly different suggesting that laminarin supplementation can alter the network interactions by changing the microbial community composition. However, laminarin post-feeding effects showed that HFD and HFL microbiota become more similar to each other than the CTL group suggesting that microbiota of HFL group shifts to obese type of microbiota similar to HFD and that laminarin could have lost its effects. A Venn diagram was generated to describe the common and unique OTUs among the three groups (Figure 12). A total of 1121 OTUs were identified from all fecal samples, with 374 of those existing in all groups defined as core OTUs. The core OTUs comprised approximately 33% of the total OTUs while 210, 206 and 173 OTUs were uniquely identified from CTL, HFD and HFL fed mice, respectively. This result suggests that species richness could be lowered by consumption of high fat diet.

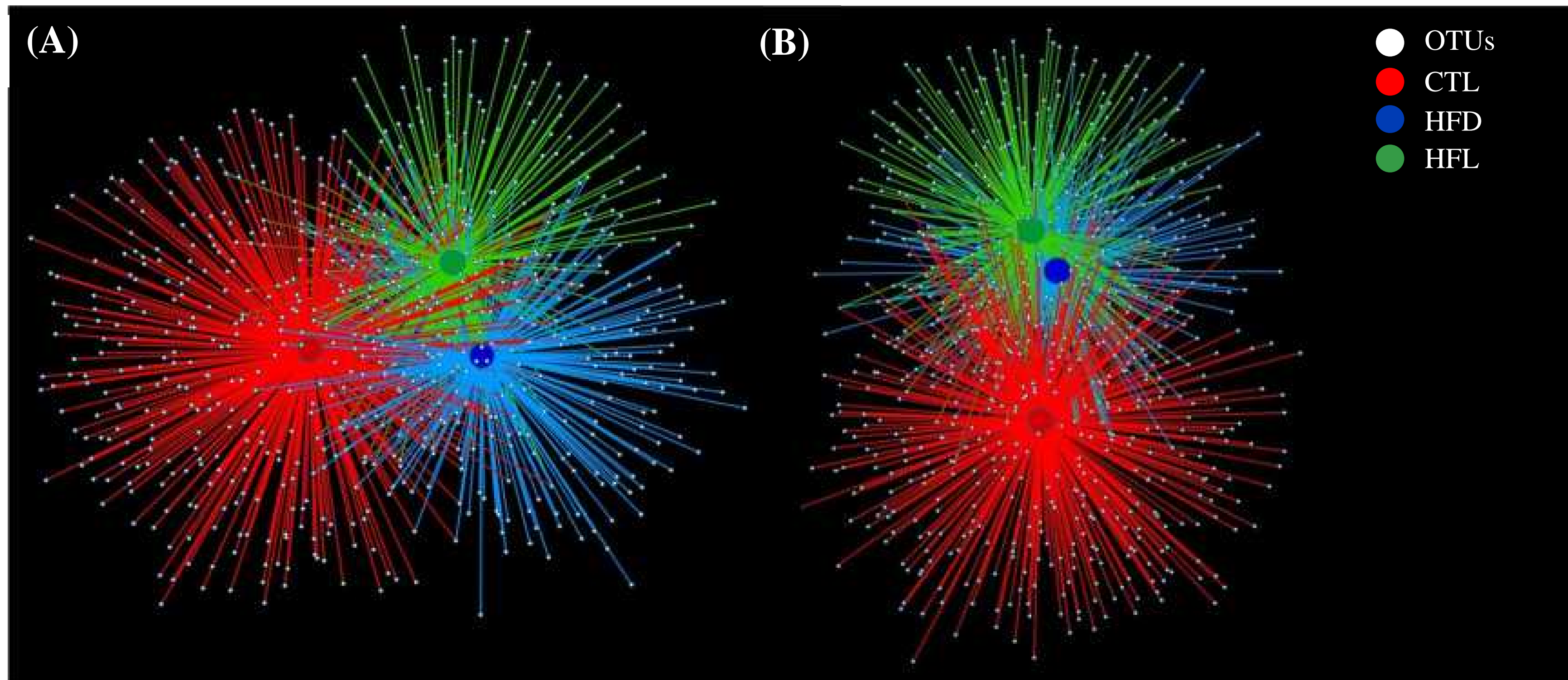

Figure 11. Network analysis of common and unique OTUs of the three experimental treatment groups (CTL, HFD and HFL) during (A) laminarin feeding and (B) laminarin post-feeding. Red, blue and green circles denote CTL, HFD and HFL fed mice group, respectively. Small black dots correspond to OTUs. Nodes represent operational taxonomic units (OTUs), and each line indicates that an OTU was identified in the same source

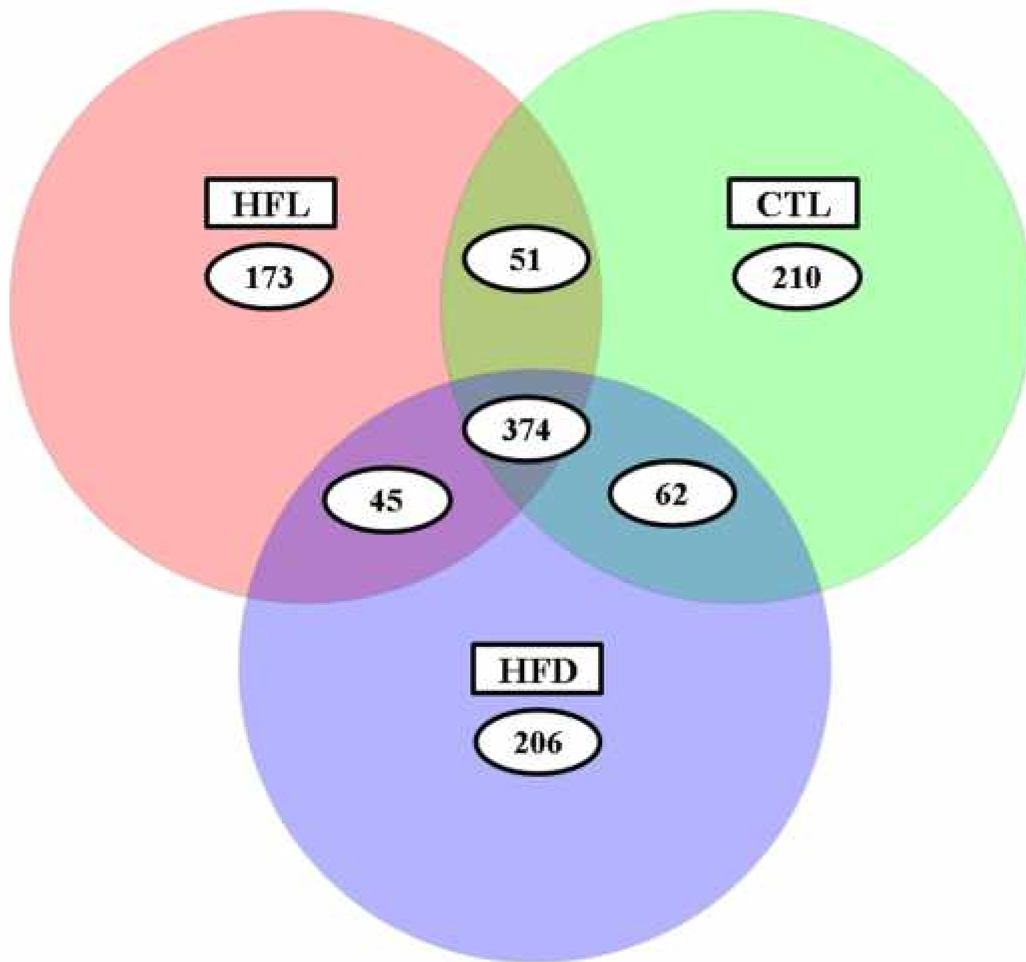

Figure 12 .Venn diagram of shared OTUs among the three treatments CTL, HFD and HFL fed mice group at 3% cutoff. Overlapping regions were drawn to scale and the number of shared and unique OTUs listed.

Effect of laminarin on fecal SCFA concentrations

Seven standard substances were used in this study: pyruvic, lactic, formic, acetic, propionic, isobutyric, and butyric acid. The calibration curves of the SCFA are shown in Figure 13. The correlation coefficient was 0.99 for all the standards. Standard samples were injected three times in different concentrations and the average retention time (Rt) was used. From the analysis, the Rt for pyruvic, lactic, formic, acetic, propionic, isobutyric and butyric acids was 10.73, 14.52, 15.79, 17.12, 19.93, 22.30, and 24.00 minutes, respectively. Figure 14 shows the proportion of individual SCFA present in the feces of CTL, HFD and HFL fed mice at week 4. Acetic, formic, and propionic acid represented major part of the SCFA produced with 85.21%, 85.34% and 78.60% in CTL, HFD and HFL group, respectively. Isobutyric acid was not detected in all the fecal samples. While there are conflicting results about fecal SCFA association with obesity, our study showed lower amount SCFA in high fat fed mice. Laminarin did not affect the amount of fecal SCFA although laminarin could be a source of SCFA. SCFA could be consumed by hosts or simply did not affect activities of probiotic gut microbes.

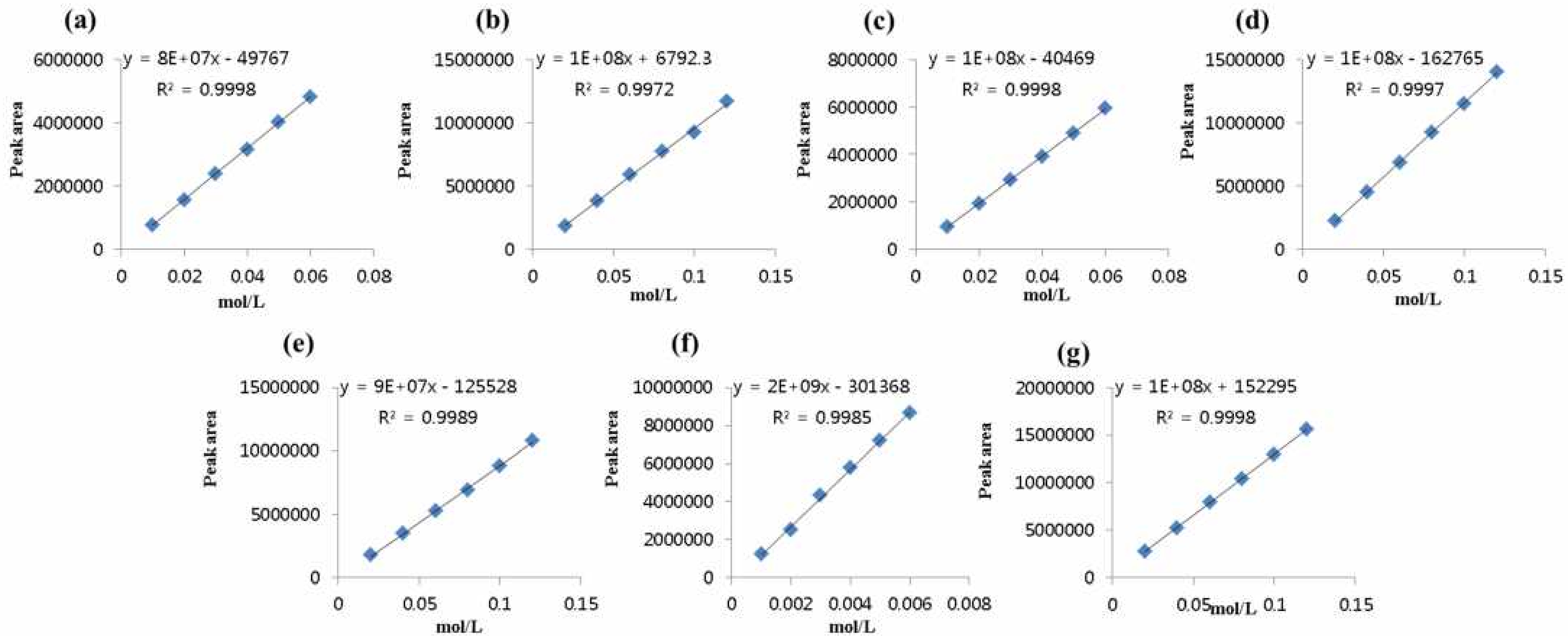

Figure 13. Standard curves of seven short-chain fatty acids used in the study: (a) acetic, (b) butyric, (c) formic, (d) lactic, (e) propionic, (f) pyruvic, (g) isobutyric acid.

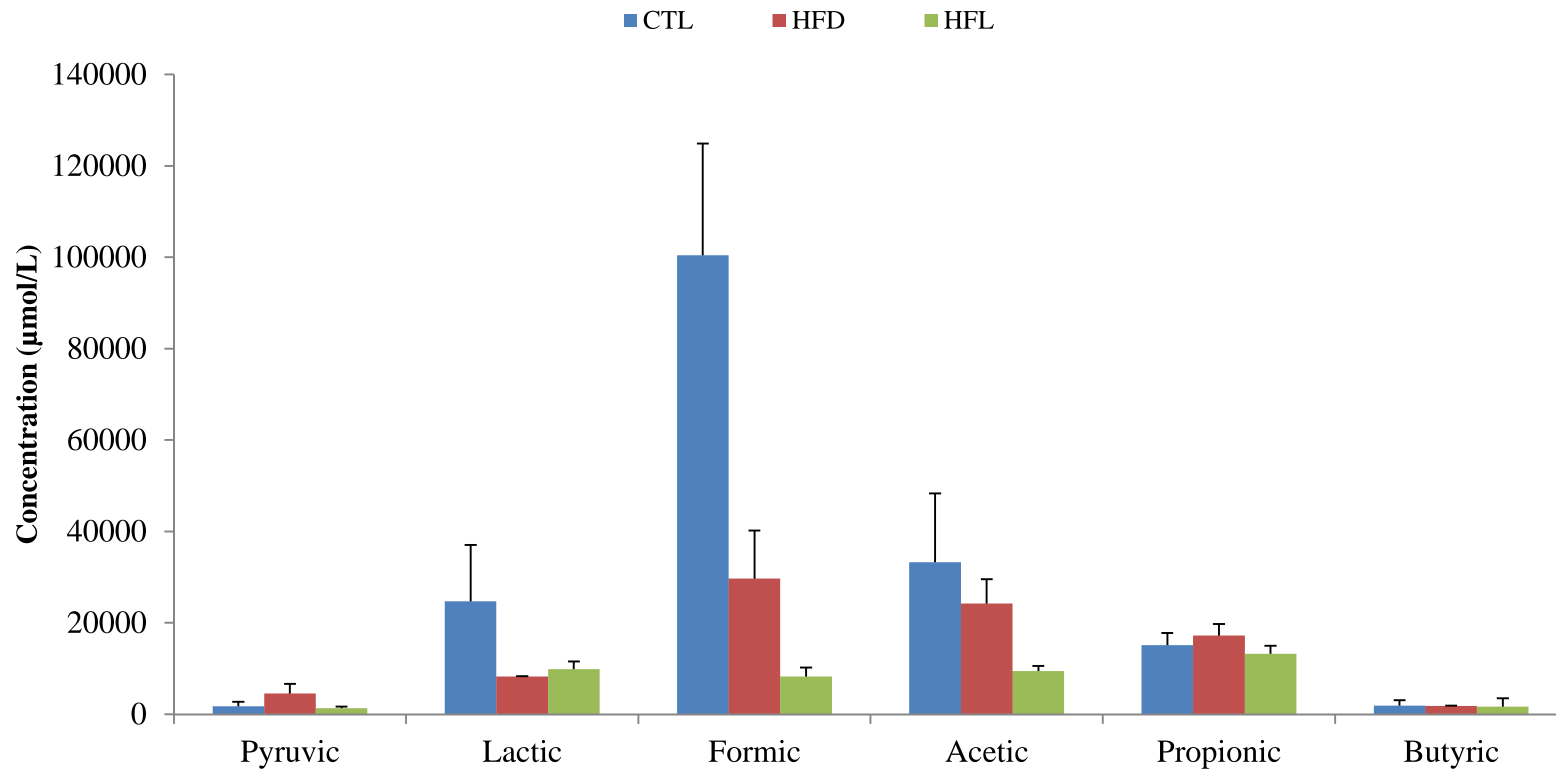

Figure 14. Concentrations of short-chain fatty acids in feces of CTL, HFD, and HFL fed mice.

CONCLUSION

In this study, we investigated prebiotics effects of laminarin as an obesity preventing functional food. Laminarin-fed mice stopped gaining weight at the fourth week, but started gaining weight after terminating laminarin ingestion. In addition, fecal microbiota obtained from diet-induced obese mice showed significant differences between laminarin-fed mice and its control. The microbiota shifts were apparently shown decrease of ratio of the phyla *Firmicutes* and *Bacteroidetes* which was previously referred to an “obese” type microbiota. In contrast, the ratio increased after the termination of laminarin ingestion, suggesting that the effect of laminarin did not last more than a week. While there are conflicting results about fecal SCFA association with obesity, our study showed lower amount SCFA in high fat fed mice. Laminarin did not affect the amount of fecal SCFA although laminarin could be a source of SCFA. SCFA could be consumed by hosts or simply did not affect activities of probiotic gut microbes. Current NGS-based microbial community analysis relies on amplification of 16S rRNA gene, causing quantification bias in results. The use of universal primers tends to amplify major bacterial communities only, therefore minor bacterial community including probiotics needs to be examined culture-based approach. Nevertheless, our results strongly support the functional characteristics of laminarin as a prebiotics. Further study should include functional metagenomics approach in order to characterize gut microbial shifts regarding to metabolic pathways.

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