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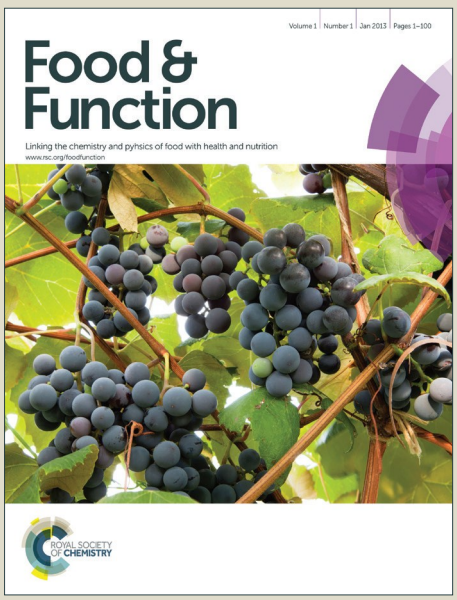

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Laminarin favorably modulates gut microbiota in mice fed a high-fat diet

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Running title: Anti-obesity effects of laminarin

Abstract

We investigated the anti-obesity effects of the potential prebiotic, laminarin, on mice fed a high-fat diet. Metagenomics approach was applied to characterize the ecological and functional differences of gut microbiota among mice fed a normal diet (CTL), high-fat diet (HFD), and laminarin-supplemented high-fat diet (HFL).

HFL mice showed slower weight gain than HFD mice during the laminarin-feeding period, but the rate of weight gain increased after the termination of laminarin supplementation. Gut microbial community analysis showed clear differences between CTL and HFD mice, whereas HFL mice were between the two. A higher abundance of carbohydrate active enzymes were observed in HFL mice compared to HFD mice, with especially notable increases in glycoside hydrolase and polysaccharide lyases. A significant decrease in Firmicutes and increase in Bacteroidetes phyla, especially the genus *Bacteroides,* were observed during laminarin ingestion. Laminarin ingestion altered gut microbiota at the species level, which was re-shifted after termination of laminarin ingestion.

Therefore, supplementing laminarin could reduce the adverse effects of a high-fat diet by shifting gut microbiota towards higher energy metabolism. Thus, laminarin could be used to develop anti-obesity functional foods. Our results also suggest that laminarin would need to be consumed regularly in order to prevent or manage obesity.

Keywords: laminarin; gut microbiota; obesity; prebiotics; metagenomics

Introduction

Obesity has become an important health concern. Since the development of sequencing technologies, a number of studies have reported on the importance of studying the gut microbiota for better understanding obesity mechanisms. The impact of gut microbiota on obesity was demonstrated in a study in which fecal bacteria from obese mice were transferred to germ-free mice and subsequently increased weight gain [1]. The authors further described that fecal microbiota in obese mice are associated with an increase in Firmicutes and decrease in Bacteroidetes, which likely increases the abundance of bacteria that code genes related to lipid and carbohydrate metabolism [2]. Moreover, Firmicutes have also been suggested to play a role in transporting sugars into cells through the phosphotransferase system [3]. In contrast, other investigators have reported no association between the Firmicutes/Bacteroidetes ratio and obesity [4-6], suggesting that the implications of obese-type gut microbiota are not yet fully understood. Nevertheless, there is increasing evidence that gut microbiota play a key role in controlling host metabolic functions.

A prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits on host wellbeing and health" [7]. This improvement includes protection against obesity. It has been reported that prebiotics can reduce gut permeability, thus providing protection against metabolic diseases [8]. In addition, the administration of prebiotics may reduce adiposity by reducing G protein-coupled receptors [9] and promoting changes in the gut microbiota associated with weight loss [10]. Therefore, treatment with prebiotics has become an effective therapy that offers protection against obesity.

Seaweeds are potential prebiotics that are rich in polysaccharides. Extracts from seaweeds have demonstrated antibacterial [11] and potential antioxidant activities [12]. There are three major polysaccharides in seaweeds: fucoidan, alginate, and laminarin. Fucoidan has been reported to inhibit lipid accumulation in adipocytes [13-15]. Alginate is also known to reduce the activity of digestive enzymes, such as pepsin and pancreatic lipase *in vitro* [16]. However, few anti-obesity effects have been reported for to laminarin. Previous studies suggest that dietary supplementation with laminarin may increase immunity against *Escherichia coli* lipopolysaccharides [17], induce TNF-alpha production from human peripheral blood monocytes [18], and increase production of short chain fatty acids, especially butyrate, in the gut, [19], which could provide an intestinal barrier protective against metabolic diseases [20]. Although these effects may not be directly linked to obesity prevention, laminarin may improve gut health and provide anti-obesity qualities.

In this study, we investigated the anti-obesity effects of laminarin on mice fed high-fat diet. We adopted a metagenomics approach to characterize the ecological and functional differences in gut microbiota of mice fed high-fat diets with or without laminarin. The study presented here provides a foundation for the future development of functional foods with laminarin.

Materials and Methods

Animals and feeding trial experiment

The animal protocols used in this study were reviewed and approved based on the ethical procedures and scientific care for animals set by the Jeju National University Institutional Animal Care and Use Committee (JNU-IACUC; Approval Number 2015-0019) in accordance

with Korean Ministry of Food and Drug Safety guideline (Laboratory Animal Act-11987). In total, 18 4 week-old female BALB/c mice were housed in stainless steel wire cages in a room maintained at 22–24°C with 55 ± 5% relative humidity and controlled lighting (12 h light/dark cycle). Animals were randomly assigned to three dietary groups (3 mice per cage, 2 cages per group) fed: i) a normal diet (CTL), ii) a high-fat diet (HFD) (45% kcal from fat, D12451 Research Diets, New Brunswick, NJ), or iii) a high-fat diet and 1% laminarin-supplemented water (HFL). Laminarin (Sigma) supplementation was terminated at the 4th week, followed by continuation HFD for an additional 2 weeks. This allowed us investigate the post-effects of laminarin supplementation. Both solid diet and water were consumed *ad libitum*. Mouse body weight was measured every two weeks, and feces were collected from each cage weekly.

16S rRNA gene and metagenome sequencing

Total DNA was extracted from 100 mg of feces collected from each cage using the MOBIO Power Fecal DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). The V4 region of the 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) using duplexed barcode fusion primers as previously described (Kozich et al. 2013). Briefly, PCR was performed using a Maxime PCR PreMix Kit (iNtRON Biotechnology Inc., Korea) as follows: 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 an AccuPrep PCR Purification Kit (Bionner Co., Korea). Equimolar PCR products were pooled and stored at −20°C until sequencing. The amplicons then were sequenced in both directions (250 bp x 2) with MiSeq (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

Replicates of DNA samples were pooled for 4-week time points from of CTL, HFD, and HFL groups and an additional 6-week time point from the HFL group (labeled CTL4, HFD4, HFL4, and HFL6, respectively). The pooled samples were separately subjected to metagenome shotgun sequencing using HiSeq 2000 (100bp x 2) according to the manufacturer's instructions. All sequencing was done at Macrogen Inc. (Republic of Korea).

Microbial community analysis

MiSeq raw reads were deposited to short read archives (SRA) with registration number SRP075347. Erroneous sequences were removed through paired-end read assembly with PEAR version 0.9.5 [21], alignment to SILVA SSU database (release 123) [22], and chimera removal using UCHIME [23]. MOTHUR [24] version 1.34.4 was used to conduct alpha and beta diversity analyses. Taxonomic classification was done using the database from the Ribosomal Database Project (release 11) [25]. Non-archaeal/bacterial lineages were removed from downstream analyses. Prior to cluster analysis, the numbers of sequences per sample were normalized based on minimum number of sequences per sample. MOTHUR average neighbor clustering was used to assign operational taxonomic units (OTUs) based on sequence identity (≥ 97%). The numbers of raw and screened reads and OTUs are summarized in Table S1.

Based on number of OTUs, species richness (ACE) and diversity indices (Shannon) were calculated using MOTHUR. Community comparison analyses were performed using non-metric multidimensional scaling (NMDS) using the MOTHUR NMDS command. Correlation between NMDS plots and abundance of taxonomic composition was analyzed using the MOTHUR corr.axes command with metadata option. Any significant correlations ($P < 0.05$) were overlaid on NMDS plots using R Arrows function. Shared OTUs were analyzed based on the common

OTUs among the replicates and applied for network analysis using Cytoscape [26]. The organic yFiles layout was applied to estimate alternation of OTUs according to time and food source.

Metagenome sequences analysis

Sequence data obtained from HiSeq paired-end sequencing were deposited at SRA with registration number SRP075339. Trimming was performed with PEARF software (option: -q 28 -f 0.25 -t 0.05 -l 30) [27] to trim low quality reads. Trimmed reads were mapped to the carbohydrate active enzymes (CAZyme) database containing families of glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), and auxiliary activities (AA) [28]. USEARCH [29] was used to map trimmed reads with e-value 1e-9 and top-hit-only options. Mappings were considered successful if both paired end reads were mapped to the same enzyme.

Statistical analysis

Significant differences between phyla and genera were examined with Tukey's HSD test. A differential abundance test was done using Metastats [30] and OTUs with $P < 0.05$ defined as differentially abundant OTUs. Pearson correlation analysis was applied to find taxa that correlated to the NMDS results. Analysis of molecular variance (AMOVA) was also applied to examine significant differences in NMDS plots.

Results

Effect of laminarin on mice weight gain

All mice weighed between 17 and 19 g at the start of the experiment (day 0), and gained 3 to 5 g by the end of the experiments (Fig. S1). Relative weight gain increase is shown in Figure 1. Feeding an HFD significantly increased relative weight gain compared to CTL ($P < 0.05$). HFD and HFL mice showed higher weight gain during the first two weeks compared to CTL mice. Then, the rate of weight gain in HFL mice dramatically decreased during the second two weeks (week 2–4). After termination of laminarin supplementation, the rate of weight gain in HFL mice has become similar to that of HFD mice.

Sequencing results

The V4 region of 16S rRNA gene PCR amplicons was sequenced using MiSeq. A total of 2,035,026 sequences were obtained from 42 fecal samples in this study (Table S1). The number of reads per sample ranged from 8,894 to 157,219. After removal of erroneous sequences, nearly 72–85% of reads remained. The number of sequences per sample was normalized based on the minimum number of reads per sample (7,144) and used for the downstream analyses. The number of operational taxonomic units (OTUs) obtained from this study ranged from 91 to 302 (Table S1).

Comparison of ecological indices

Ecological indices (ACE and Shannon) were calculated and are shown in Figure 2. Significant differences in species richness (ACE) were observed between CTL and HFD-HFL mice ($P < 0.05$) during the entire feeding trial (Fig. 2A). In addition, species richness in HFL mice was even lower than in HFD mice during laminarin ingestion, but no difference was observed after termination of laminarin ingestion (Fig. 2B). This suggests that laminarin

ingestion lowered species richness. On the other hand, no significant difference was observed in species diversity (Shannon) between CTL and HFD mice, or between HFD and HFL mice during the entire feeding trial (Fig. 2C, D). However, HFL mice showed significantly lower species diversity compared to CTL mice during the laminarin ingestion period, suggesting that laminarin lowered species diversity as well.

Comparison of taxonomic composition

Taxonomic compositional differences are shown in Figure 3. Feeding a high-fat diet increased Actinobacteria and decreased Tenericutes during the entire feeding trial. On the other hand, significant differences between HFD and HFL mice were found in abundances of Firmicutes, Bacteroidetes, and Proteobacteria phyla. The abundance of Proteobacteria was significantly higher in HFL during the entire feeding trial, whereas the abundance of Bacteroidetes was increased and Firmicutes was decreased only during the laminarin supplementation period. This suggests that the abundances of the two phyla are sensitive to ingestion of laminarin. During laminarin supplementation, the abundance of *Parabacteroides* and *Bacteroides* significantly increased more in HFL mice than in HFD mice ($P < 0.05$). On the other hand, feeding an HFD significantly decreased the number of *Clostridium_XIVa* in HFD mice ($P < 0.05$), but not in HFL mice during laminarin supplementation. In contrast, an HFD significantly increased abundance of *Clostridium_XIVb* and *Clostridium_XI* ($P < 0.05$), but decreased their abundance in HFL mice ($P < 0.05$). None of these shifts in specific genera was observed after termination of laminarin supplementation (Fig. S2).

Distribution analysis of operational taxonomic units

The correlation analysis between the distribution of OTUs of each sample and abundance of each phylum is shown in Figure 4. Significant separations between dietary groups ($P < 0.001$) were observed during laminarin ingestion (Fig. 4A). CTL and HFD groups were most distant from each other (Bray-curtis 0.675), whereas HFL was in the middle between HFD and CTL groups. HFD samples showed a positive correlation with the abundance of Firmicutes and Deferribacteres bacteria ($P < 0.05$). In contrast, abundance of Bacteroidetes was negatively correlated to that of Firmicutes. Both high-fat diet groups (HFD and HFL) exhibited negative correlations with abundance of Tenericutes *($P < 0.05$)*. After the termination of laminarin supplementation, the microbial compositions in HFL samples became similar to those from HFD samples, with higher abundance of Firmicutes and lower abundance of Bacteroidetes (Fig. 4B).

Shared and unshared OTUs were analyzed based on network analysis (Fig. 5). During laminarin ingestion, network analysis positioned all CTL samples and Day 0 of both HFD and HFL samples together, whereas week 1–4 HFD and HFL samples were separately positioned (Fig. 5A), suggesting that alternation of OTUs had dynamically occurred after only one week into the feeding trial, and was maintained during the entire feeding period. However, the difference between HFL and HFD samples was shortened when 5–6 week samples were included (Fig. 5B).

During laminarin ingestion, differential abundance tests found 180 abundant OTUs (38,616 reads) in HFD samples and 81 abundant OTUs (43,756 reads) in HFL samples (Table S2). Among the differentially abundant OTUs in HFD mice, genus *Clostridium* XI (1 OTU, 5.8 %) presented as the most abundant, whereas *Bacteroides* (4 OTUs, 51.6%) followed by *Clostridium* XVIII (1 OUT, 5.3%) were the most abundant in HFL mice. These results suggest that *Bacteroides* were selectively increased by the laminarin ingestion. In contrast, far fewer

OTUs were found differentially abundant across HFD and HFL mice after termination of laminarin supplementation: 15 OTUs (4,360 reads) and 13 OTUs (1,880 reads) in HFD and HFL samples, respectively. This indicates that the influence of laminarin on abundance of specific species was lost after termination of laminarin supplementation (Table S3).

Carbohydrate active enzyme distribution analysis

Metagenome sequences were obtained from week 4 samples for all experimental groups (CTL4, HFD4, and HFL4) and from the week 6 sample for the HFL group (HFL6). More than 26 million metagenomic reads were obtained from each sample. Metagenomic reads were normalized based on the minimum number of reads (23,026,795). Mapping results are summarized in Table 1. The highest numbers of mapped sequences were observed for HFL4, followed by HFL6. The HFD4 sample had the lowest numbers in each family of CAZymes, whereas nearly 2–3 times more mapped sequences were detected from HFL4. Among the CAZyme families found, Glycoside Hydrolases (GHs) were the most abundant, followed by Glycosyl Transferases (GTs). Almost the same amounts of Carbohydrate Esters (CEs) and Polysaccharide Lyases (PLs) were found, and Auxiliary Activities (AAs) were found by far the least. The specific families that were most increased during laminarin ingestion were GH2, GT2, GT4, PL1, PL10, CE8, and CE12 (Fig. S3).

Discussion

An HFD has been used many times to induce obesity in mice. HFDs significantly lower species richness, which further causes adverse effects to host physiological functions [31]. Although ingestion of laminarin did not increase species richness, it did shift gut microbial

communities. Studies have shown that consumption of an HFD increases Firmicutes species and decreased Bacteroidetes [1, 2]. Since energy harvesting capacity is mostly controlled by CAZymes produced by many of Bacteroidetes species [32], the reduced number of Bacteroidetes associated with HFD subsequently causes a decrease in energy harvesting capacity within the gut. Moreover, correlation analyses in previous studies indicate that certain bacteria are strongly associated with gut health. For example, although most *Clostridium* species are commensal, some are pathogenic, such as *Clostridium difficile* that belongs to cluster XI [33] and those in *Clostridium* cluster XIVb, which is known to be associated with metabolic syndrome [34]. Nevertheless, certain *Clostridium* species are considered beneficial, such as those in cluster XIVa, which can colonize mucins and produce butyrate [35]. Other beneficial microbes include *Bacteroides*, whose genomes encode many CAZymes [36], and *Parabacteroides* that modulate immunity [37]. In this study, we observed that supplementation with laminarin decreased the abundance of potentially pathogenic bacteria (*Clostridium* cluster XIVb and XI), potentially reversing the adverse effects caused by HFD-induced structural changes in gut microbiota. Likewise, ingestion of laminarin was correlated with increases in beneficial bacteria (*Clostridium* cluster XIVa, *Parabacteroides* and *Bacteroides*). Most notably, we observed a dramatic increase in *Bacteroides,* subsequently leading to an increase in CAZymes, as well. A total of 12 OTUs were classified to the genus *Bacteroides*, but differential abundance analysis only detected 4 OTUs of *Bacteroides* that were significantly increased in HFL, thus suggesting that laminarin selectively increases only certain species of *Bacteroides*.

Among the increased CAZymes associated with HFL, we observed that the numbers of GH2, GT2, GT4, PL1, PL10, CE8, and CE12 were especially increased compared to other families. GH2 is involved in hemicellulose degradation [38] and degradation of the pectin side

chain [39], thus playing a key role in digestion of dietary polysaccharides in the gut. GT is far less diverse compared to GH [40], and GT2 and GT4 are prototypes of GT [41]; thus many reads were mapped to these two families. It has been reported that beta cell-specific GT is effective in reducing the chance of developing diabetes [42]. GT2 is involved in chitin synthesis [43], which is known to reduce body mass index [44]. Therefore, increased amounts of GT may prevent obesity. Like GH, PL functions in degrading polysaccharides. PL1 and PL10 are pectate lyases, in which different folds of the proteins carry identically poised catalytic machinery that perform the same reaction on the same substrate [45]. PL1 is abundant in green roughage fed animals [46], suggesting that the enzyme plays a key role in digesting prebiotics and increasing energy harvesting capacity. CE hydrolyzes ferulic acid and *O*-acetyl esters, which aid in hemicellulose degradation and contribute to complete hydrolysis of polysaccharides [47]. Pectin methyl esterase (CE8) and acetyl esterase (CE12) are highly expressed in the human gut symbiont, *Bacteroides xylanisolvens* [48], suggesting that these enzymes may be highly expressed in response to consumption of dietary fiber. In addition, it has been reported that polysaccharides in green tea extracts are responsible for its anti-obesity effects on diet-induced obese rats [49]. Moreover polysaccharides also show lipid lowering effects in high cholesterol-fed mice [50].

Recent studies have shown that consuming seaweed-based diets can enrich PL [51] and GH [52] in the human gut microbiome. In addition, it has been reported that microbial glycan degradation plays a key role in diet-driven adaptation of gut microbiota [53-55]. Thus, a laminarin-supplemented diet could shift gut microbiota by increasing bacteria that digest dietary polysaccharides and decreased potentially pathogenic bacteria. In our study, weight loss in HDL mice started after two weeks of laminarin ingestion. Dynamic species (OTUs) alteration was observed a week later. It has been reported that dietary changes shift the gut microbiota within 3

293 days and can shift back after returning to the original diet [56]. Therefore, supplementation of
294 laminarin would need to be continued in order to maintain its anti-obesity effects. Here, we
295 report that laminarin has a high potential to be defined as a prebiotic. The presented study will be
296 informative for the development of functional foods with laminarin.
297

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304

305 **Conflict of Interest**

306 There is no conflict of interest to declare.

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481 Table 1. Number of metagenome reads per million that have high similarity to carbohydrate
482 active enzymes. HFD4, HFL4, HFL6, and CTL4 denote each dietary group and week of samples:
483 HFD, high-fat diet; HFL, high-fat diet with laminarin; and CTL, control diet.

484

Families of carbohydrate active enzymes	HFD4	HFL4	HFL6	CTL4
AA	3	16	3	0
CE	239	557	393	319
GH	5,037	10,569	7,772	6,395
GT	1,285	2,299	1,786	1,341
PL	162	432	320	249
Total	6,726	13,873	10,274	8,304

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Figure 1: Rate of weight gain (%) of mice fed a control diet (CTL), high-fat diet (HFD), and high-fat diet supplemented with laminarin (HFL).

Figure 2: Comparison of species richness (A, C) and diversity (B, D) in gut microbiota from mice fed different diets before (A, B) and after the termination of laminarin feeding (C, D): CTL, normal diet; HFD, high-fat diet; and HFL high-fat diet supplemented with laminarin. Superscripts 'a', 'b', and 'c' indicate significant difference ($P < 0.05$).

Figure 3: Log scale abundance analysis at the phylum (A) and genus levels (B) during the laminarin feeding period. 'A' and 'B' differentiate cages.

Figure 4: Non-metric multidimensional scaling analysis and correlation of taxonomic abundance at the phylum level during the laminarin feeding period (A) and after termination of laminarin supplementation (B). CTL, HFD, and HFL denote groups of mice fed control diet, high-fat diet, and high-fat diet supplemented with laminarin, respectively. Ellipses were drawn to denote 95^{th} percentiles.

Figure 5: Network analysis of operational taxonomic units during laminarin ingestion (A) and entire feeding period (B). Colors indicate sampling time and shapes indicate dietary treatments: diamond, control diet; square, high-fat diet; and triangle, high-fat diet supplemented with laminarin.

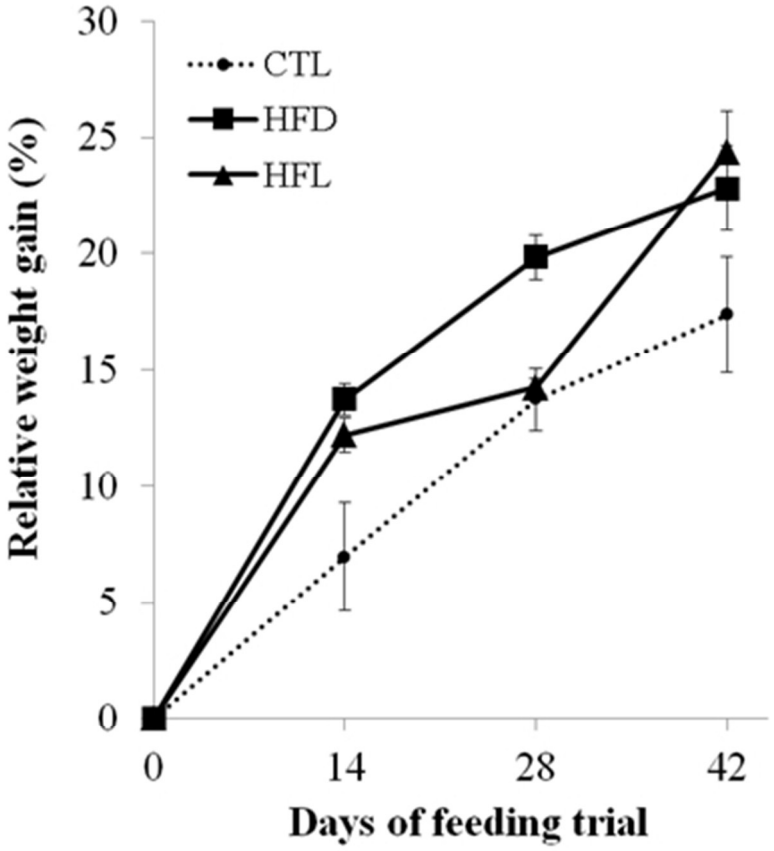

Figure 1: Rate of weight gain (%) of mice fed a control diet (CTL), high-fat diet (HFD), and high-fat diet supplemented with laminarin (HFL).

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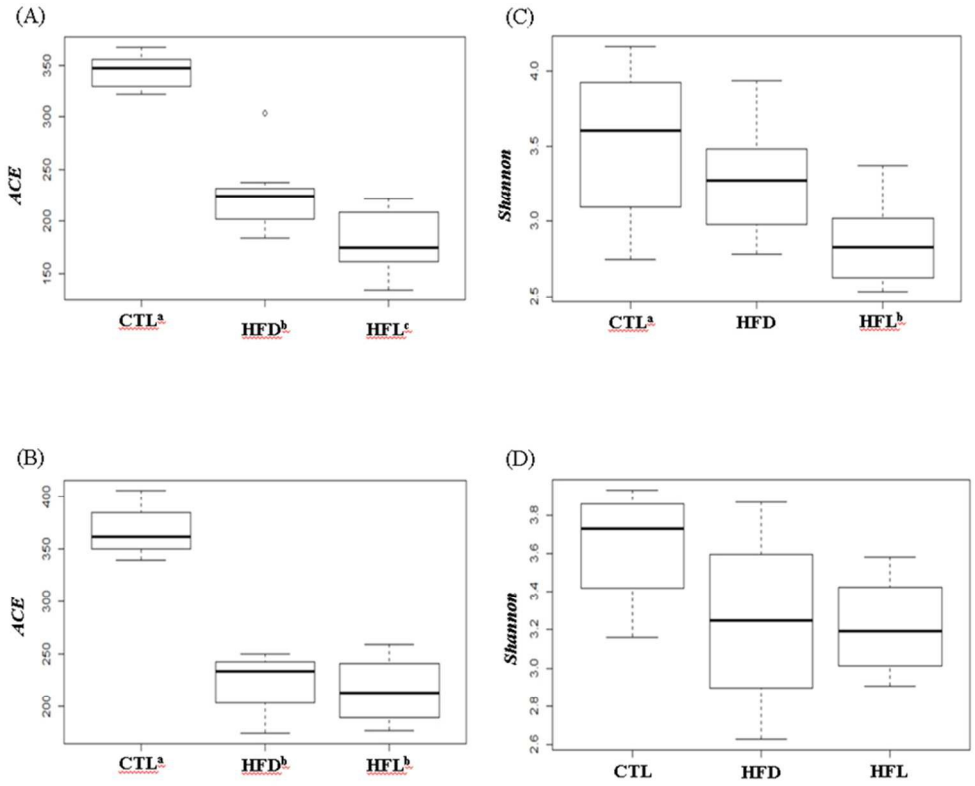

Figure 2: Comparison of species richness (A) and diversity (II) in gut microbiota from mice fed different diets: CTL, normal diet; HFD, high-fat diet; and HFL high-fat diet supplemented with laminarin. Superscripts 'a', 'b', and 'c' indicate significant difference ($P < 0.05$).

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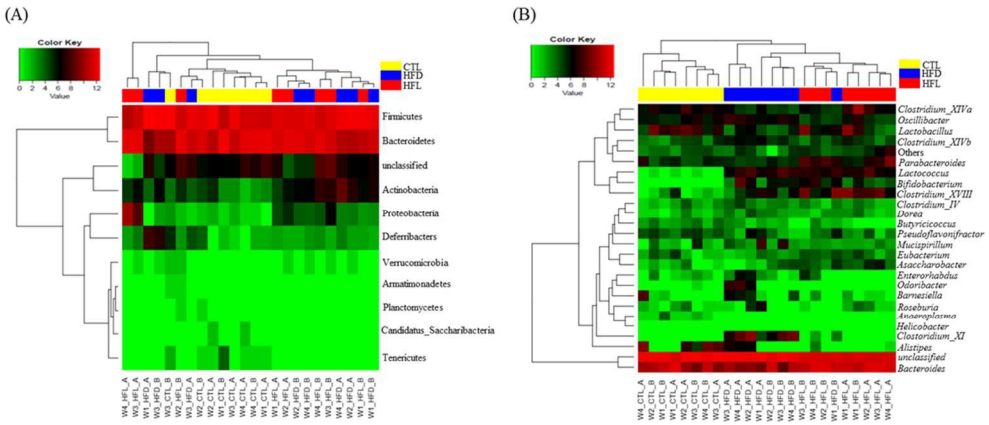

Figure 3: Log scale abundance analysis at the phylum (A) and genus levels (B) during the laminarin feeding period. 'A' and 'B' differentiate cages.

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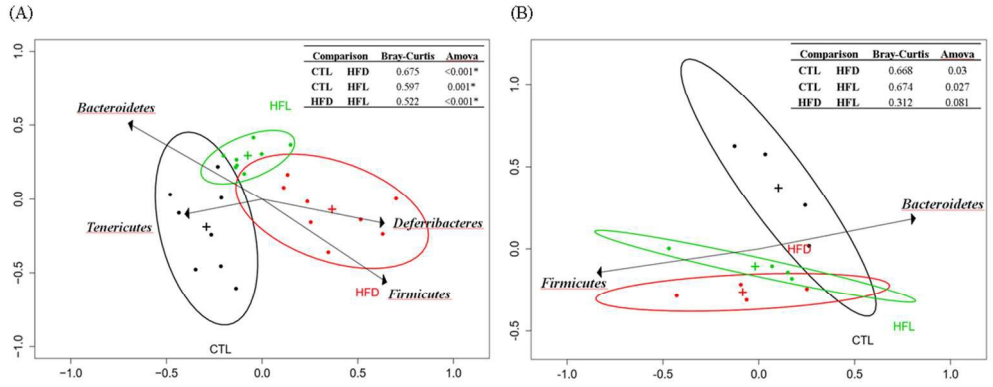

Figure 4: Non-metric multidimensional scaling analysis and correlation of taxonomic abundance at the phylum level during the laminarin feeding period (A) and after termination of laminarin supplementation (B). CTL, HFD, and HFL denote groups of mice fed control diet, high-fat diet, and high-fat diet supplemented with laminarin, respectively. Ellipses were drawn to denote 95th percentiles.

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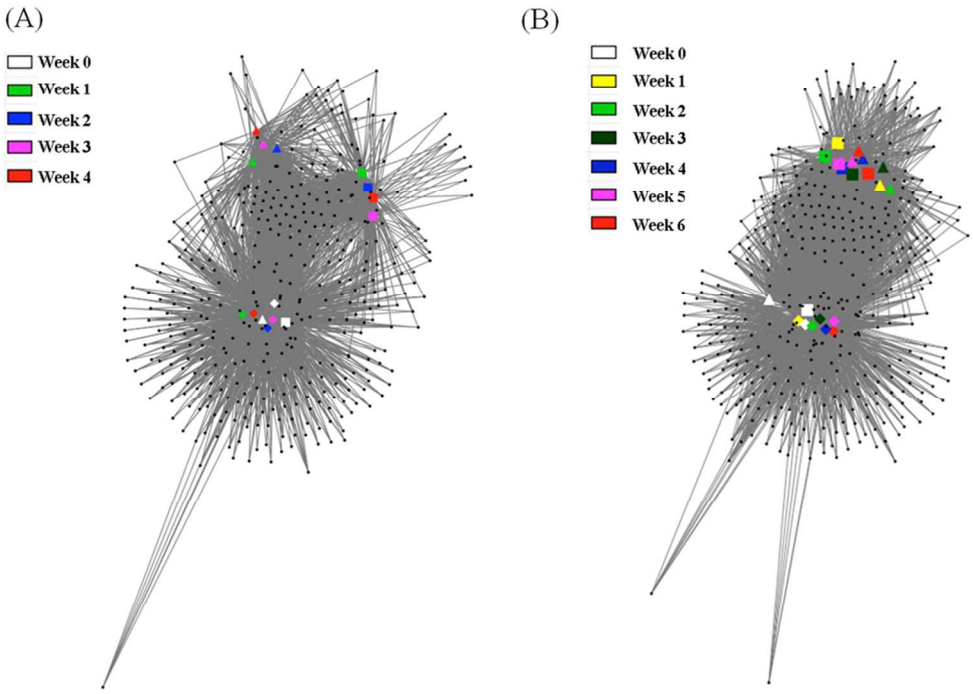

Figure 5: Network analysis of operational taxonomic units during laminarin ingestion (A) and entire feeding period (B). Colors indicate sampling time and shapes indicate dietary treatments: diamond, control diet; square, high-fat diet; and triangle, high-fat diet supplemented with laminarin.

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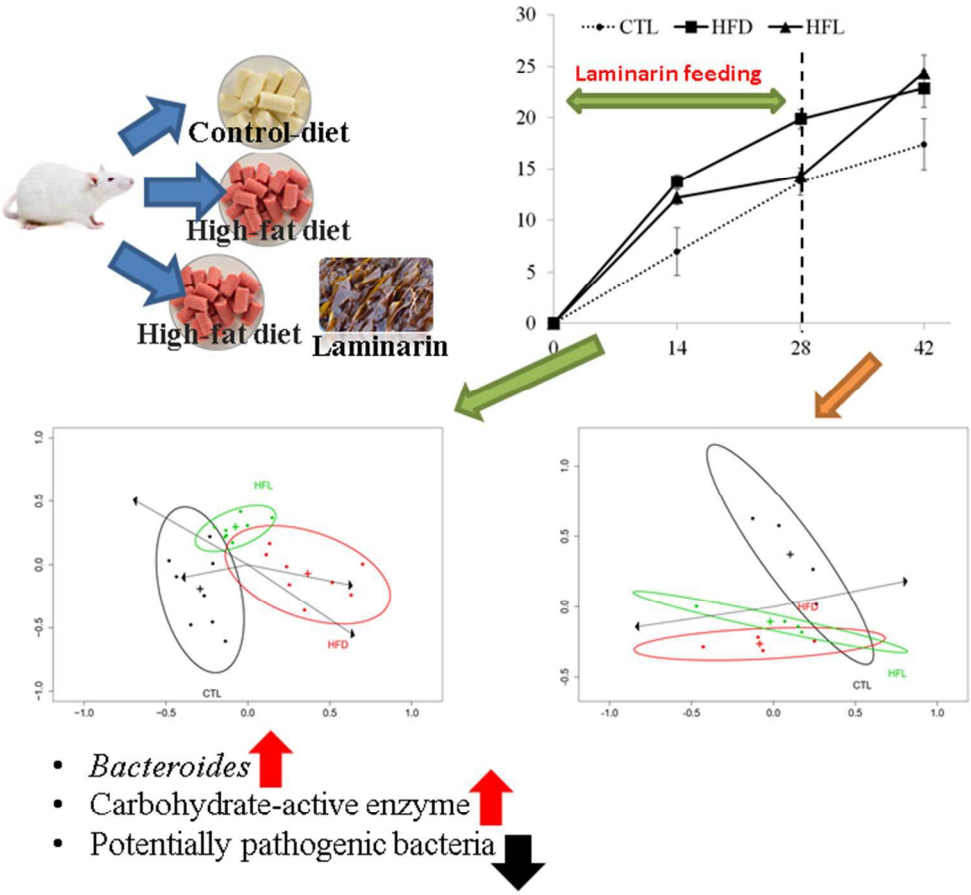

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