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Microbiome dynamic modulation through functional diets based on pre- and probiotics (mannan-oligosaccharides and *Saccharomyces cerevisiae*) in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Running tittle: **Fish microbiome fed functional feed**

Abstract

Aims

This study used high-throughput sequencing to evaluate the intestinal microbiome dynamics in rainbow trout (*Oncorhynchus mykiss*) fed commercial diets supplemented with either pre- or probiotics (0.6% mannan-oligosaccharides and 0.5% *Saccharomyces cerevisiae* respectively) or the mixture of both.

Methods and Results

A total of 57 fish whole intestinal mucosa and contents bacterial communities were characterized by high-throughput sequencing and analysis of the V3-V4 region of the 16S rRNA gene, as well as the relationship between plasma biochemical health indicators and microbiome diversity. This was performed at 7, 14 and 30 days after start feeding functional diets, and microbiome diversity increased when fish fed functional diets after 7 days and it was positively correlated with plasma cholesterol levels. Dominant phyla were, in descending order, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Fusobacteria*. However, functional diets reduced the abundance of *Gammaproteobacteria* to favor abundances of organisms from *Firmicutes* and *Fusobacteria*, two phyla with members that confer beneficial effects. A dynamic shift of the microbiome composition was observed with changes after 7 days of feeding and the modulation by functional diets tend to cluster the corresponding groups apart from CTRL group. The core microbiome showed an overall stability with functional diets, except genus such as *Escherichia-Shigella* that suffered severe reductions on their abundances when feeding any of the functional diets.

Conclusions

Functional diets based on pre- or probiotics dynamically modulate intestinal microbiota of juvenile trout engaging taxonomical abundance shifts that might impact fish physiological performance.

Significance and Impact of the Study

This study shows for the first time the microbiome modulation dynamics by functional diets based on mannan oligosaccharides and *S. cerevisiae* and their synergy using culture independent high-throughput sequencing technology, revealing the complexity behind the dietary modulation with functional feeds in aquatic organisms.

Keywords Microbiome, Rainbow trout, Metagenomics, Functional diets, Prebiotics, Probiotics, *Saccharomyces cerevisiae*, mannan-oligosaccharides

Introduction

Microbiota are key to regulating health in higher vertebrates (Belkaid and Hand 2014, Kelder *et al.* 2014) and bony fish (Gómez and Balcázar 2008). Microbes colonizing animal intestines act as another "organ," performing important functions unfulfilled by the host (O'Hara and Shanahan 2006). The intestinal microbiota participates in nutrient digestion and metabolism (Semova *et al.* 2012, Gatesoupe *et al.* 2014), in the immune response (Gómez and Balcázar 2008, Mansfield *et al.* 2010), in appetite and regulating ingestion (Marques *et al.* 2014), and in regulating gene expressions (Dalmasso *et al.* 2011). These regulatory roles are especially relevant under culture conditions, when animals can often experience homeostatic disruptions as a result of surroundings deviating from norms established in the wild. Aquaculture practices, in particular, repeatedly expose fish to a variety of deleterious events (e.g. changing water quality, nutritional alterations, high stocking densities, therapeutic applications, etc.) that severely impact the microbiome (Olsen *et al.* 2002, Olsen *et al.* 2005). For example, Olsen et al. (2005) found that a 15 min acute chasing event significantly reduces the intestinal microbiota in rainbow trout (*Oncorhynchus mykiss*). Disruptions in microbiota balance, or dysbiosis, negatively affect organisms, and although microbiome research in fish is a recent research area, reports have already associated dysbiosis with disease outbreaks (Llewellyn *et al.* 2014).

Fish gut microbiomes are shaped by environmental conditions (De Roy *et al.* 2013), feeding habits (Desai *et al.* 2012, Ingerslev *et al.* 2014, Li *et al.* 2014), species (Sullam *et al.* 2012), and life cycle stage (Desai *et al.* 2012), and the last decade has witnessed increased investigations on microbiome modulation. While several factors modulate gut microbiota, diet-related regulations are a focal point of research (Ringø *et al.* 2016) since dietary manipulations and optimization are a primary strategy for improving fish development in aquaculture. Related to this, Desai et al. (2012) found that rainbow trout fed diets enriched with plant-meal rather than fish-meal proteins presented significant changes in the distal gut microbiome, reflecting possible health issues for fish. Furthermore, Li et al. (2014) compared the gut microbiomes of eight fish species with different feeding habits and found dominant taxa-level changes correlated with blood biochemistry, suggesting that microbiota may influence physiological processes. Interestingly, Bolnick et al. (2014) studied the effect of dietary diversity on the gut microbiomes of the threespine stickleback (*Gasterosteus aculeatus*) and Eurasian perch (*Perca fluviatilis*), finding a negative correlation in which dietary specialists (*G. aculeatus*) had greater microbial diversity than dietary generalists (*P. fluviatilis*).

Functional diets that can enhance fish health and improve disease resistance are one of the most highly demanded types of nutritional intervention in aquaculture. In general, functional diets are defined as natural or processed feeds that contain known or unknown biologically-active compounds in amounts clinically proven to promote health traits that aid in disease prevention or treatment (Ferguson 2009). Ringø et al. (2016) recently presented a thorough review for the effects of dietary components in aquatic animal gut microbiota, specifically providing support for the microbiome modulatory capacities of different lipids, proteins, carbohydrates, vitamins, amino acids, and several dietary supplements included in functional feeds. Of the several compounds under study, the main actors in functional feed due to health-improvement and immunostimulatory abilities are prebiotics, which are indigestible compounds that promote healthy microbiota growth/host immunostimulation, and probiotics, which are live microorganisms that confer host health benefits when administered in sufficient concentrations (Merrifield *et al.* 2010, Lauzon *et al.* 2014, Ringø and Song 2016). The underlying action mechanisms of pre- and probiotics are still under study (Zorriehzahra *et al.* 2016). Some features known to positively contribute to intestinal microbiota (Denev *et al.* 2009, Merrifield and Carnevali 2014) include a competition for mucosal layer attachment sites (Vine *et al.* 2004), the production of inhibitory substances with bactericidal or bacteriostatic actions, competition for available chemicals and energy (De *et al.* 2014, Zorriehzahra *et al.* 2016), and even interfering with quorum sensing (Fuente *et al.* 2015).

Considering the existing literature, the aim of this study was to apply cutting-edge high-throughput sequencing technologies towards improving understandings on microbiota modulation by functional diets in fish. A metagenomics approach was applied to evaluate microbiome dynamics in *O. mykiss* intestines after being fed with diets supplemented by either pre- or probiotics. Overall, the results evidence the complexity of the fish microbiome and how functional diets can serve as a powerful modulatory tool to improve fish aquaculture worldwide.

Material and Methods

Experimental Design and Sampling Procedures

All animals used in this study were treated in accordance to the Biosecurity Regulations and Ethical Protocols approved by Universidad de Concepción Ethics Committee and the CONICYT-Chile.

Freshwater-cultured sexually immature juvenile rainbow trout (*O. mykiss*, n = 120) with an average weight (± SD) of 28.3 ± 2.8 g were obtained from a commercial fish farm located in the Los Lagos Region of Chile. Fish were screened for health conditions and certified free of the most prevalent pathogens prior to experiments. The fish were transferred to a recirculating freshwater system (450 L total volume), and individuals were randomly divided in triplicate (per diet) between tanks (37 L) reaching a density of 7.8 Kg m^{-3}. Fish were acclimated for 14 d to a 12:12 h light:dark photoperiod, 13 ± 0.5 °C, pH 7.5, and ammonium levels were below detection limits, and water quality was monitored to maintain these conditions. Fish were fed once daily with a commercial diet (Ewos®, Coronel, Chile) in proportion to 1.3% of the total biomass for each tank.

After acclimatization, fish were fed for 30 d with functional diets (1.2% proportion of biomass; Table 1). Three functional diets were formulated, a prebiotic diet (PRE; addition of mannan-oligosaccharides), a probiotic diet (PRO; addition of *Saccharomyces cerevisiae*), and a mixed pre/probiotic diet (MIX; addition of mannan-oligosaccharides and *S. cerevisiae*). The supplements, together with an oil mixture (supplied by Ewos®), were incorporated into dry commercial pellets by vacuum coating at the Ewos® experimental feed unit (Coronel, Chile). A control diet (CTRL) was also prepared following the same protocol, with dry commercial pellets incorporating the oil mixture but no supplements.

All formulated pellets were kept refrigerated and dry throughout the experimental period, and the total feed biomass given to fish was adjusted weekly following sampling, thereby accounting for decreased biomass due to fish sampling, as well as increased biomass due to the weight gained by non-sampled fish (calculated using the sampled fish as indicators). Fish behavior was carefully observed during feeding to ensure that groups presented a stable appetite and no feed rejection. To verify *S. cerevisiae* viability and concentration in the probiotic and mix-coated pellets, a suspension (1 g) of each pellet type was prepared weekly in sterile phosphate saline buffer, serially diluted, inoculated in a Sabouraud agar medium, and finally incubated for 48 h at 30 ºC before posterior counting of colony forming units. The concentrations of *S. cerevisiae* in diets ranged between 3.9×10^4 and 4.7×10^4 c.f.u g^{-1} of feed from PRO and MIX, whereas PRE and CTRL group present no yeast growth under the tested conditions.

Fish (n = 3 per tank) were randomly sampled 7, 14, and 30 d after the start of the functional diet trials (Additional file 1: Figure S1). All sampled fish were anesthetized with clove oil (0.3 mL 10% eugenol L^{-1}), weighed, and sized, and blood (≈ 1 mL) was collected from the caudal vein with 0.1% EDTA-coated syringes. In a disinfected surface (with 70% ethanol) with a permanent flame, fish were then euthanized by cervical dislocation, surface cleaned with 70% ethanol, and the peritoneal cavity anatomized. Then, the whole intestines were carefully dissected, snap frozen in liquid nitrogen, and stored at -80 ºC. Before sampling fish were starved for 24 hours, therefore digesta was not found in the intestines of any specimen and sample included intestinal tissue and mucus. At each sampling point one more fish per tank was sampled for evaluation of the quantity of *S. cerevisiae* remained active in intestines. This was performed by dissecting the whole intestine, weighted and suspending in PBS as above described for feed. Along the experimental trial concentrations of *S. cerevisiae* in intestines of PRO and MIX groups ranged from $1.1\text{x}10^4$ and $2.3\text{x}10^4$ c.f.u g^{-1} of tissue, whereas in PRE and CTRL groups ranged between 50 and 211 c.f.u g^{-1} tissue. Blood samples were centrifuged (10 min, 1500 x *g*) to collect plasma, which was stored at -20 ºC until biochemistry analysis. Between each dissection, instruments were disinfected by washing with 70% alcohol followed by 10% chloride and ultrapure water.

Blood Biochemistry

Plasma levels were measured with commercial kits from Human Diagnostics (Wiesbaden, Germany), including for glucose (Glucose LiquiColor), total cholesterol (Cholesterol LiquiColor), triglycerides (Triglycerides $\text{LiquiColor}^{\text{mono}}$), gamma glutamyl transpeptidase (Gamma-GT LiquiColor), and alanine (GPT [ALAT] IFCC mod. liquiUV) and aspartate transaminases (GOT [ASAT] IFCC mod. liquiUV), following the manufacturer's instructions. These kits were previously validated as suitable for rainbow trout metabolite and enzyme measurements.

DNA Extraction and 16S rRNA Sequencing

Total genomic DNA was obtained from whole intestinal samples by thawing the samples, using a sterile scalpel to scrape internal contents and mucosa, and applying the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, USA) following the manufacturer's protocol. The integrity of each DNA sample was evaluated using agarose gel electrophoresis and quantification in a Qubit v2.0 fluorometer (Life Technologies, Darmstadt, Germany). Prior to library preparation, universal primers (515F sequence GTGCCAGCMGCCGCGGTAA, and 806R sequence GGACTACHVGGGTWTCTAAT) were used to PCR assess each sample to ensure the presence of bacterial DNA following Caporaso et al. (2012), and samples that did not present amplicon or had dubious amplicon band were eliminated from subsequent analysis. PCR was performed in a Verity Thermal Cycler (Applied Biosystems, Thermo Scientific, USA) and the amplification conditions were as follows: 94 °C for 3 min, 35 cycles at 94 °C for 45 s, 50 ºC for 60 s, and 72 °C for 90 s and 72°C for 10 min. Samples qualifying for metagenome sequencing were sent to Macrogen (Seoul, Korea) for further processing (57 samples in total, Supplementary Table S1). Libraries were constructed using the Illumina MiSeq Platform, with 16S rRNA gene amplicons prepared following the manufacturer's instructions. Briefly, the bacterial V3 and V4 variable regions of the 16S ribosomal gene were amplified for each sample from template DNA (60 ng) using Illumina primers and barcoded adapters, following the PCR conditions recommended by Illumina. Samples were pooled in equal proportions and pair-end sequenced through 300 cycles in a MiSeq Platform by Macrogen (Seoul, Korea). PhiX viral DNA was added to the sample pool as a control for focusing, template building, and phasing, thus mitigating possible problems arising from the sequencing of a low diversity library, such as 16S rRNA.

Metagenomics Analysis

Paired-end reads were demultiplexed, and Illumina adaptors were removed by Macrogen's in-house pipeline before being reported in FASTQ format. The paired-end sequences for each sample were merged using FLASH (Magoc and Salzberg 2011) and a minimum overlap threshold of ten base pairs. Data were further processed using the Quantitative Insights into Microbial Ecology (QIIME) bioinformatics pipeline. The resulting sets of merged reads were pre-processed to trim low quality (threshold phred score of 33) and chimeric reads, and general denoising was applied using CD-HIT-OUT (Li *et al.* 2012). The remaining representative, non-chimeric reads were clustered into operational taxonomic units (OTUs) using a 97% identity threshold. The reads were taxonomically classified through matching against sequences in the Greengenes database (version gg_13_5). Matching was performed in QIIME using the UCLUST algorithm (Edgar 2010). Coverage percentage was estimated by Good's nonparametric coverage estimator method; alpha diversity was evaluated with a bias-corrected Chao1 richness estimator; and the Shannon and Simpson diversity indices were calculated through Mothur (Schloss *et al.* 2009). Microbial community structures in different samples were compared using UniFrac based on the phylogenetic relationship of representative reads from different samples, and the weighted UniFrac distances were used to build a principal coordinate analysis.

Statistical Analysis

Data from blood biochemistry and condition factor were screened for normality, and variables not distributed normally were Box-Cox transformed. Differences between the effects of the four diets, in triplicate, were tested with a two-way ANOVA, followed by a post-hoc Tukey's HSD test when applicable. Differences between alpha diversity indices were evaluated with the non-parametric test Wilcoxon signed rank test pair by pair at each time. Correlation analysis between diversity indices and biochemical parameters was performed to infer possible physiological relationship. A two-way permutational multivariate analysis of variance (PERMANOVA) was performed with 999 permutations for the weighted UniFrac distance matrix that was obtained by beta diversity analysis. This was used to test the hypothesis that there were no differences between the intestinal microbiota communities when fish were fed functional diets along a 30 days trial. All statistical analysis was performed using JMP 9.0 software package (SAS Institute Inc., Cary, NC), except for the PERMANOVA that was performed with CLC Genomics workbench version 9 with Microbial Module (Qiagen Bioinformatics, USA). For microbiome communities descriptive assessment, only taxa that presented a cumulative 1 % abundance in at least one group were included in subsequent analysis.

Results

Biochemical Plasma Parameters

No mortalities occurred during the experimental period, nor were differences in Fulton's condition factor (K) found (Table 2). Plasma glucose levels decreased in PRE fish after 14 d but increased in PRE and MIX fish after 30 d. These results indicate significant dietary effects on this parameter (Table 2). Plasma triglyceride levels were highest in MIX fish at 7 and 30 d, whereas changes in total cholesterol were not significant between diets or sampling days. However, gamma glutamyl transpeptidase plasma levels were lower in the PRE group after 7 d and in the PRE and MIX groups after 14 d, as compared to the PRO and CTRL diets. Similarly, lower aspartate transaminase levels were recorded in PRE and MIX fish after 14 d. Finally, alanine transaminase levels were lower in the MIX group after 14 d and in the PRE group after 30 d.

Sequencing

Paired end merge by FLASH resulted in 4,629,576 reads distributed by the 57 samples (Additional file 2: Table S1), after processing raw reads and filtering for quality and ambiguities, Illumina MiSeq analysis returned a total of 929,062 reads from the 57 intestine samples (Additional file 3: Table S2). Ten samples presented < 1,000 filtered reads and were discarded from further analyses, thus controlling for any possible technical issues during processing. A total of 324 different OTUs were identified from the 47 remaining samples. Good's nonparametric coverage estimator method indicated 98.6 ± 0.007% diversity recovery per sample. All samples reached a saturation plateau, as shown by rarefaction curves constructed from OTUs (Fig. 1A). Therefore, sequencing contained most of the bacterial species present.

Richness and Diversity

Chao1 richness estimates were not different between groups throughout the experimental period, excepting the PRE group, which evidenced significantly higher Chao1 richness after 30 d of feeding as compared to the CTRL and PRO groups (Fig. 1B). In turn, both the Shannon and Simpson indices (Figure 1C, D, respectively) showed a significant increase in intestinal bacterial community diversity for all functional diets after 7 d, remaining elevated in the PRE group over the entire feeding trial. Both indices were significantly affected by diet

(P < 0.01), with higher diversity in the functional diet groups when comparing with control group.

Correlation analyses between biochemical plasma parameters and alpha diversity indices obtained no significant results, with the exception of total plasma cholesterol levels, which were positively correlated with the number of OTUs and with Chao1 richness (Pearson's correlation coefficients of 0.44 and 0.47 with P = 0.02 and P = 0.001, respectively). Furthermore, beta diversity analysis, represented by a principal coordinate analysis based on weighted UniFrac distances, showed two main groups, indicating similarities between CTRL replicates at all sampling points (Fig. 2). Clusters were formed by the PRO groups after 30 d and MIX groups after 14 d. In turn, the PRE group presented similarities with the MIX group after 7 and 30 d and with the PRO group after 7 and 14 d. PERMANOVA analysis revealed that both time and diet produced an effect on intestinal microbial communities (Table 3), and contrast analysis (Table 4S) corroborated most of the differences observed by principal coordinate analysis indicating a separation of the microbial communities of the three CTRL groups together with PRO 30d and MIX 14d from the communities of all the other functional diets groups.

Microbiome modulation at taxonomic levels

The dominant bacterial phyla identified in all CTRL samples were, in descending order, *Proteobacteria* (75%), *Firmicutes* (14.6%), *Actinobacteria* (2.3%), *Acidobacteria* (2%), *Bacteroidetes* (1.8%), and *Fusobacteria* (0.6%) (Fig. 3). The functional diet groups evidenced a tendency of greater relative abundances of phyla other than *Proteobacteria*, such as *Firmicutes*, *Bacteroidetes*, and *Fusobacteria*. As an exception, the PRO group showed notably high *Proteobacteria* abundances (84%) after 30 d. In PRO, PRE, and MIX groups, the average relative abundances of *Firmicutes* increased to 16.7%, 20.7%, and 16.3%, respectively, over the experimental period. Similarly, *Bacteroidetes* abundances respectively increased to 4%, 8%, and 5.3%, while *Fusobacteria* increased to 1.4%, 2.8%, and 1.2%.

The dynamics of the modulation of Classes in fish fed functional diets is represented in Additional File 2: Figure S2 *Gammaproteobacteria* abundances seemed to be reduced in the MIX group after 7 d as compared to the CTRL group, whereas there was an increase in the abundances *Alphaproteobacteria* in the MIX group, *Fusobacteriales* in the PRE group, *Betaproteobacteria* in the PRO and MIX groups, and *Bacilli* in all functional diet groups. In all functional diets, *Clostridium* abundances increased in comparison with CTRL group's community, especially after 30 d, a sampling point at which CTRL group *Clostridium* abundances did not reach 1%. Microbial community composition in fish fed functional diets seemed influenced by trial duration, with changes occurring over time.

Two main clusters (Fig. 4) resulted from the hierarchical clustering of distances obtained from order-level abundances. One cluster was composed by the CTRL (7, 14, and 30 d), MIX (14 d), and PRO (14 d) groups. The second cluster included the remaining groups, all of which were functional diet groups. The two clusters were separated by order-level abundance averages. The first cluster was mostly loaded by high *Enterobacteria* abundances, followed by several additional taxa, such as *Burkholderiales*, *Fusobacteriaceae*, *Pseudomonadales*, and *Bacillales*.

Regarding the number of shared and unique OTUs per diet at each sampling time (Fig. 5A), the CTRL group had a higher number of exclusive OTUs after 7 and 14 d. However, after 30 d, the number of OTUs shared with the MIX group increased. Among all groups, the PRE and MIX groups shared the highest quantity of OTUs from 14 d to the end of the experiment. No groups presented exclusive OTUs at all sampling points. Therefore, while exclusive OTUs were identified after 7, 14, or 30 d, these were not necessarily the same OTUs at all times. In contrast, consistently shared OTUs among all diet groups were identified and taxonomically characterized (Fig. 5B). Specifically, OTUs for 28 taxa were found, comprising the core microbiota of rainbow trout in this study. The identified taxa strains included five *Alphaproteobacteria*, two *Betaproteobacteria*, and seven *Gammaproteobacteria*. The seven *Gammaproteobacteria* strains were distributed among the genera *Escherichia-Shigella*, *Moraxella*, *Haemophylus*, *Pseudomonas, Serratia*, and *Acinetobacter*. Furthermore, four *Firmicutes* phylum strains were found, three of which were from the *Streptococcus* genus. Similarly, three *Actinobacteria* strains; five *Acidobacteria* strains, with four from the DA052 family; and one *Bacterioidetes* strain identified as a *Porphyromonas* were recorded.

Stabilities for the noted core microbiota were evaluated through fold-changes in each group as compared to the CTRL group. While the abundances of most taxa changed between 1- and 2-fold, some had higher abundancy shifts. Specifically, *Escherichia-Shigella* abundance was greatly reduced by functional diets, with the exceptions of PRO and MIX after 14 d. *Acidovorax* had increased abundances after 7 d, followed by a reduction after 14 d before again increasing at 30 d. In contrast, *Rhodoplanes* were less abundant after 7 d but more abundant after 14 d. Similarly, the two *Streptococcus* strains underwent reduced abundances after 7 d, but were much more abundant after 14 d. Generally, these two strains were more abundant in the functional diet groups as compared to the CTRL group after 30 d, with the exception of the PRO group, in which one of the *Streptococcus* strains evidenced 8-fold lower abundances.

Discussion

The microbiome of rainbow trout intestinal mucosa and contents is a dynamic environment that can be quickly, but not permanently, modulated by functional diets based on pre- and probiotics. Details on the modulatory capacities of these functional ingredients were elucidated in the present study by using high-throughput sequencing to identify microbiome taxa in fish fed different diets over 30 d. A core microbiota was identified that showed some resilience against the modulatory actions of the functional diets, but several beneficial exceptions were identified.

Several reports exist in fish regarding the microbiota-modulating effects of dietary modifications (revised by (Merrifield and Carnevali 2014) and (Ringø *et al.* 2016), but the vast majority of these studies were based on culture-dependent techniques, thus limiting results. Indeed, the intestinal microbiome of fish is highly complex and dynamic, as only recently revealed by PCR-based microbial analyses (Desai *et al.* 2012, Guerreiro *et al.* 2015). In aquaculture, pre- and probiotics have been used to modulate fish intestinal microbiomes due to various beneficial actions, including an ability to promote a healthy microbiota, thus improving host condition (Zorriehzahra *et al.* 2016). However, deciphering the interplay between functional ingredients and autochthonous microbiota requires the application of newer molecular techniques that can accurately and reliably produce sequence datasets by acquiring a large quantity of sequence reads. One such technology is high-throughput sequencing, which, to date, has been scarcely applied in the context of fish microbiota modulation by diet. To this end, the present study successfully applied high-throughput sequencing to the 16S V3 and V4 regions, and these assays identified structural shifts in the microbial community of rainbow trout intestinal mucosa and contents after being fed diets supplemented with either prebiotics (i.e. mannan-oligosaccharides), probiotics (i.e. *S. cerevisiae*), or a synergetic mix of pre- and probiotics. To our knowledge, this is the first in-depth report on microbiome composition in fish fed with diets supplemented by *S. cerevisiae*.

The presently observed increase in intestinal microbiome diversity in fish fed pre- and probiotic supplemented diets, as measured by the Shannon and Simpson diversity indices, was in line with that found by other researchers. Dimitroglou et al. (2010) reported that dietary mannan-oligosaccharides increase microbial gut diversity in gilthead seabream (*Sparus aurata*). This result was also obtained by Waché et al. (2006) after feeding rainbow trout fry with a *S. cerevisiae* supplemented diet. In contrast, Falcinelli et al. (2016) recently reported reduced microbial diversity in zebra fish (*Danio rerio*) larvae fed with probiotic *Lactobacillus rhamnosus* GG for 8 d, but this was mainly associated with potential pathogenic taxa being limited by increased abundances of beneficial microbiota. In general, a diverse microbiome is indicative of community stability and proper functioning, translating into better adaptive capacities when faced with deleterious events, such as stress or pathogenic invasion (Kassen and Rainey 2004, De Roy *et al.* 2013). The present findings suggest that PRE and PRO fish have a healthier microbial community, which should be reflected in the long-term by better fish health.

The microbe-host health connection is well reported in vertebrates (Albenberg and Wu 2014, Belkaid and Hand 2014), including bony fish. Intestinal bacteria have such an important role in host physiological balance that any dysbiosis can have severe effects. Metabolic, immune, digestive and neuronal processes are regulated by intestinal microbiota (Gómez and Balcázar 2008, Belkaid and Hand 2014, Marques *et al.* 2014), and probiotic modulations towards a healthier state can promote physiological robustness in fish. For example, Falcinelli et al. (Falcinelli *et al.* 2015) recently identified a relationship between lower microbial diversity and lower plasma cholesterol/triglycerides in zebrafish after being fed *L. rhamnosus*. Interestingly, the present analyses positively correlated plasma cholesterol and microbial community Chao1 richness, suggesting lipid metabolism modulation via microbiome richness. The observed increases in plasma glucose and triglyceride levels in the PRE and MIX groups are indicative of improved nutritional statuses, where these fish would have more energy resources available for rapid use against any homeostatic impairment (Peres *et al.* 2014). These results were likely influenced by overall microbial community diversity and consequently better health arising from improved digestion and nutrient utilization (Nayak 2010). While Fulton's condition factor (K) was not altered by the tested functional diets, the ranges presented by fish were generally good. This observation was further supported by reduced plasma enzyme activities in PRE and MIX fish, which is indicative of hepatic stability and proper functioning (Peres *et al.* 2014). Future studies will evaluate how functional diets impact the condition index during the rainbow trout culture production cycle (up to 18 months), as well as if functional diets work better or more efficiently during specific periods.

Despite the fact that this study was performed in a controlled environment, fish were maintained under recirculating conditions. Consequently, one concern for analyses was if the duration of the experimental period could have affected intestinal microbiome compositions. This concern was discarded after evaluating beta diversity results, which showed all CTRL group replicates clustered in the same group, indicating phylogenetically similar microbiome communities. Furthermore, phyla/class community compositions in the CTRL groups remained rather stable over time, which was in contrast with the more dynamic microbiomes of the functional diet groups. The most dominant phyla in CTRL fish were consistent with previous reports for rainbow trout (Pond *et al.* 2006, Mansfield *et al.* 2010, Navarrete *et al.* 2010) and Atlantic salmon (*Salmo salar*) (Gajardo *et al.* 2016). Nevertheless, while the functional diet groups had the same dominant phyla, pre- and probiotic supplements promoted a redistribution of abundances towards *Firmicutes*, *Bacteroidetes*, and *Acidobacteria*. Increased *Firmicutes* abundances were also observed by Falcinelli et al. (2016) in trials where zebrafish larvae were fed the probiotic *L. rhamnosus* GG. Regarding the class-level changes induced by functional diets, not only did *Proteobacteria* abundances decreased, but the abundances of the three main classes also shifted. Specifically, *Alpha*- and *Betaproteobacteria* abundances increased while the relative abundances of

Gammaproteobacteria decreased. *Gammaproteobacteria* include the most pathologically important Gram-negative bacteria, particularly from the families *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae*. Therefore, a reduced abundance of phylum members indicates an effect of potentially pathogenic microorganisms. In the short-term (7 d), abundances of the *Bacilli* and *Clostridia* classes also increased in the intestines of functional diet fish. These classes include most of the known beneficial bacteria used as probiotics, such as *Bacillus* sp., *Lactobacillus* sp., *Leuconostoc* sp. *Streptococcus* sp., *Staphylococcus* sp., *Carnobacterium* sp., and *Enterococcus* sp., among others (Romero *et al.* 2014). In turn, *Clostridium* species are frequently isolated from freshwater fish and are recognized enzyme producers (Ray *et al.* 2012) or probiotics, as in the case of *Clostridium butyricum* (Pan *et al.* 2008). Interestingly, all functional diet groups showed increased abundances of *Clostridia*. The PRE group also evidenced increased *Fusobacteria*, which are Gram-negative bacilli that can produce butyrate, a short-chain fatty acid. In mammals, butyrate is beneficial, providing energy to gastrointestinal cells or enhancing mucus production (Hamer *et al.* 2009), whereas in fish, butyrate improves growth and physiological function (Robles *et al.* 2013). However, *Fusobacteria* have scarce abundance in carnivorous species such as rainbow trout (Wong *et al.* 2013), therefore, dietary mannan-oligosaccharides could increase *Fusobacteria* abundances, granting the host benefits of the biological functions performed by this class as seen by Burr et al. (2010). Much of the research on pre- and probiotic supplemented diets has focused on the positive modulation of *Bacilli* class members (Hoseinifar *et al.* 2014, Adel *et al.* 2016) which are beneficial Gram-positive bacteria (Ringø *et al.* 2016). While the presently obtained results did evidence increases in *Bacilli* members, differences were not significant for any specific bacterium.

The interplays existing between different microbiome species in fish and other vertebrates remain unknown and should be considered in future studies due to possible health enhancing effects. Order-level microbial abundances were clustered into two clades, one of which represented nearly all mannan-oligosaccharide experimental groups (i.e. all PRE and MIX groups, excepting the MIX group at 14 d). This clustering was also detected by principal coordinated analysis of the weighted UniFrac distances between each community, as well as by the increasing number of shared OTUs between PRE and MIX groups over the experimental period. In particular, this clustering was mainly based on a reduction of *Enterobacteriales* (*Gammaproteobacteria*), a modest increase of *Lactobacillales*, and increases in *Clostridiales*, *Bacillales,* and, to lesser extents, *Pseudomonadales*, *Rickettsiales*, and other orders with potentially pathogenic members, such as *Flavobacteriales*. Relative abundance increases in these types of bacteria are not necessarily connected with pathologies since mannan-oligosaccharides can activate mechanisms of intestinal protection to reduce bacterial translocation (Torrecillas *et al.* 2011). These mechanisms might be linked to the functional capacities of each species and to respective roles in microbiota interplays, but this hypothesis requires further investigation.

This study identified a core microbiota that included 28 taxa, 12 of which are also found in the core rainbow trout microbiota reported by Wong et al. (2013). However, while Wong et al. (2013) did not report dietary effects on the core microbiota, such effects were found in the current study. Members of the genus *Escherichia-Shigella* were severely affected by functional diets, with dramatic decreases in relative abundances starting at 7 d as compared to CTRL groups. Although this effect was seen for all diets, excepting PRO and MIX groups at 14 d, the PRE group presented the most stable effect. Gram-negative bacteria, such as *Escherichia* sp., present exterior carbohydrate-binding proteins associated with antigen recognition and fimbrial adhesins. Among these, lectins attach to oligosaccharide components of glycoconjugate receptors binding to enterocytes, and type-1 fimbrial adhesins, common to many pathogenic bacteria, present specificity to mannan residues (Spring *et al.* 2000, Newman *et al.* 2006). Therefore, mannan-oligosaccharides could act as potential receptor analogs for this adhesins, thereby preventing colonization by these bacteria (Spring 2003, Newman *et al.* 2006).

In conclusion, the present study detected modulations in the intestinal microbiota of juvenile rainbow trout fed with functional diets based on pre- or probiotics. Whole intestinal mucosa and intestinal contents were evaluated together, but considering a recent study by Gajardo et al. (2016), in which dominant microbiota taxa significantly varied in Atlantic salmon depending on the analyzed intestinal section, future studies should be performed in distinct microbial compartments. Functional diets based on prebiotic mannan-oligosaccharides and probiotic *S. cerevisiae* modulated intestinal microbiota, with physiological parameters indicating these modulations to be beneficial to animal health. Specifically, the assessed functional diets reduced *Gammaproteobacteria* abundances while increasing the relative abundances of other phyla, such as *Firmicutes* and *Fusobacteria*, many members of which exercise beneficial functions in hosts.

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Ethical Statement

All animals used in this study were treated in accordance to the Biosecurity Regulations and Ethical Protocols approved by Universidad de Concepción Ethics Committee as a mandatory part of the Postdoctoral FONDECYT Grant 3140183 granted by the CONICYT-Chile.

Availability of Data and Materials

Sequencing results are available in the Sequence Read Archive (SRA) database at NCBI (Project Number PRJNA343213) and are also available from the corresponding author upon request.

Conflict of Interests

No conflict of interest declared by the authors.

References

Adel, M., Lazado, C. C., Safari, R., Yeganeh, S. and Zorriehzahra, M. J. (2016) Aqualase®, a yeast-based in-feed probiotic, modulates intestinal microbiota, immunity and growth of rainbow trout Oncorhynchus mykiss. *Aquac Res*, 10.1111/are.13019**,** 1-12.

Albenberg, L. G. and Wu, G. D. (2014) Diet and the intestinal microbiome: associations, functions, and implications for health and disease. *Gastroenterology* **146,** 1564-72.

Belkaid, Y. and Hand, T. W. (2014) Role of the microbiota in immunity and inflammation. *Cell* **157,** 121-41.

Bolnick, D. I., Snowberg, L. K., Hirsch, P. E., Lauber, C. L., Knight, R., Caporaso, J. G. and Svanback, R. (2014) Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch). *Ecol Lett* **17,** 979-87.

Burr, G., Hume, M., Ricke, S., Nisbet, D. and Gatlin, D. (2010) In Vitro and In Vivo Evaluation of the Prebiotics GroBiotic®-A, Inulin, Mannanoligosaccharide, and Galactooligosaccharide on the Digestive Microbiota and Performance of Hybrid Striped Bass (Morone chrysops × Morone saxatilis). *Microbial Ecol* **59,** 187-198.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G. and Knight, R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* **6,** 1621-4.

Dalmasso, G., Nguyen, H. T., Yan, Y., Laroui, H., Charania, M. A., Ayyadurai, S., Sitaraman, S. V. and Merlin, D. (2011) Microbiota modulate host gene expression via microRNAs. *PLoS One* **6,** e19293.

De, C. B., Meena, D. K., Behera, B. K., Das, P., Das Mohapatra, P. K. and Sharma, A. P. (2014) Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiol Biochem* **40,** 921-971.

De Roy, K., Marzorati, M., Negroni, A., Thas, O., Balloi, A., Fava, F., Verstraete, W., Daffonchio, D. and Boon, N. (2013) Environmental conditions and community evenness determine the outcome of biological invasion. *Nat Commun* **4,** 1383.

Denev, S., Staykov, Y., Moutafchieva, R. and Beev, G. (2009) Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. *Int Aquat Res* **1,** 1-29.

Desai, A. R., Links, M. G., Collins, S. A., Mansfield, G. S., Drew, M. D., Van Kessel, A. G. and Hill, J. E. (2012) Effects of plant-based diets on the distal gut microbiome of rainbow trout (Oncorhynchus mykiss). *Aquaculture* **350–353,** 134-142.

Dimitroglou, A., Merrifield, D. L., Spring, P., Sweetman, J., Moate, R. and Davies, S. J. (2010) Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (Sparus aurata). *Aquaculture* **300,** 182-188.

Edgar, R. C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26,** 2460-2461.

Falcinelli, S., Picchietti, S., Rodiles, A., Cossignani, L., Merrifield, D. L., Taddei, A. R., Maradonna, F., Olivotto, I., Gioacchini, G. and Carnevali, O. (2015) Lactobacillus rhamnosus lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. *Sci Rep* **5,** 9336.

Falcinelli, S., Rodiles, A., Unniappan, S., Picchietti, S., Gioacchini, G., Merrifield, D. L. and Carnevali, O. (2016) Probiotic treatment reduces appetite and glucose level in the zebrafish model. *Sci Rep* **6,** 18061.

Ferguson, L. R. (2009) Nutrigenomics approaches to functional foods. *J Am Diet Assoc* **109,** 452-8.

Fuente, M. L., Miranda, C. D., Jopia, P., Gonzalez-Rocha, G., Guiliani, N., Sossa, K. and Urrutia, H. (2015) Growth inhibition of bacterial fish pathogens and quorum-sensing blocking by bacteria recovered from chilean salmonid farms. *J Aquat Anim Health* **27,** 112-22.

Gajardo, K., Rodiles, A., Kortner, T. M., Krogdahl, A., Bakke, A. M., Merrifield, D. L. and Sorum, H. (2016) A high-resolution map of the gut microbiota in Atlantic salmon (Salmo salar): A basis for comparative gut microbial research. *Sci Rep* **6,** 30893.

Gatesoupe, F.-J., Huelvan, C., Le Bayon, N., Sévère, A., Aasen, I. M., Degnes, K. F., Mazurais, D., Panserat, S., Zambonino-Infante, J. L. and Kaushik, S. J. (2014) The effects of dietary carbohydrate sources and forms on metabolic response and intestinal microbiota in sea bass juveniles, Dicentrarchus labrax. *Aquaculture* **422,** 47-53.

Gómez, G. D. and Balcázar, J. L. (2008) A review on the interactions between gut microbiota and innate immunity of fish. *FEMS Immunol Med Mic* **52,** 145-154.

Guerreiro, I., Enes, P., Rodiles, A., Merrifield, D. and Oliva-Teles, A. (2015) Effects of rearing temperature and dietary short-chain fructooligosaccharides supplementation on allochthonous gut microbiota, digestive enzymes activities and intestine health of turbot (Scophthalmus maximus L.) juveniles. *Aquacult Nutr*, 10.1111/anu.12277**,** n/a-n/a.

Hamer, H. M., Jonkers, D. M., Bast, A., Vanhoutvin, S. A., Fischer, M. A., Kodde, A., Troost, F. J., Venema, K. and Brummer, R. J. (2009) Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. *Clin Nutr* **28,** 88-93.

Hoseinifar, S. H., Sharifian, M., Vesaghi, M. J., Khalili, M. and Esteban, M. A. (2014) The effects of dietary xylooligosaccharide on mucosal parameters, intestinal microbiota and morphology and growth performance of Caspian white fish (Rutilus frisii kutum) fry. *Fish Shellfish Immunol* **39,** 231-6.

Ingerslev, H. C., von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M. and Madsen, L. (2014) The development of the gut microbiota in rainbow trout (Oncorhynchus mykiss) is affected by first feeding and diet type. *Aquaculture* **424–425,** 24-34.

Kassen, R. and Rainey, P. B. (2004) The ecology and genetics of microbial diversity. *Annu Rev Microbiol* **58,** 207-31.

Kelder, T., Stroeve, J. H. M., Bijlsma, S., Radonjic, M. and Roeselers, G. (2014) Correlation network analysis reveals relationships between diet-induced changes in human gut microbiota and metabolic health. *Nutr Diabetes* **4,** e122.

Lauzon, H. L., Dimitroglou, A., Merrifield, D. L., Ringø, E. and Davies, S. J. (2014) Probiotics and Prebiotics: Concepts, Definitions and History. In Aquacult Nutr, ed. *169-184.* John Wiley & Sons, Ltd.

Li, J., Ni, J., Li, J., Wang, C., Li, X., Wu, S., Zhang, T., Yu, Y. and Yan, Q. (2014) Comparative study on gastrointestinal microbiota of eight fish species with different feeding habits. *J Appl Microbiol* **117,** 1750-60.

Li, W., Fu, L., Niu, B., Wu, S. and Wooley, J. (2012) Ultrafast clustering algorithms for metagenomic sequence analysis. *Brief Bioinform* **13,** 656-68.

Llewellyn, M., Boutin, S., Hoseinifar, S. H. and Derome, N. (2014) Teleost microbiomes: progress towards their characterisation, manipulation and applications in aquaculture and fisheries. *Front Microbiol* **5**.

Magoc, T. and Salzberg, S. L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27,** 2957-63.

Mansfield, G. S., Desai, A. R., Nilson, S. A., Van Kessel, A. G., Drew, M. D. and Hill, J. E. (2010) Characterization of rainbow trout (Oncorhynchus mykiss) intestinal microbiota and inflammatory marker gene expression in a recirculating aquaculture system. *Aquaculture* **307,** 95-104.

Marques, T. M., Cryan, J. F., Shanahan, F., Fitzgerald, G. F., Ross, R. P., Dinan, T. G. and Stanton, C. (2014) Gut microbiota modulation and implications for host health: Dietary strategies to influence the gut–brain axis. *Innov Food Sci Emerg* **22,** 239-247.

Merrifield, D. L. and Carnevali, O. (2014) Probiotic Modulation of the Gut Microbiota of Fish. In Aquacult Nutr, ed. *185-222.* John Wiley & Sons, Ltd.

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S. J., Baker, R. T. M., Bøgwald, J., Castex, M. and Ringø, E. (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302,** 1-18.

Navarrete, P., Magne, F., Mardones, P., Riveros, M., Opazo, R., Suau, A., Pochart, P. and Romero, J. (2010) Molecular analysis of intestinal microbiota of rainbow trout (Oncorhynchus mykiss). *FEMS Microbiol Ecol* **71,** 148-56.

Nayak, S. K. (2010) Role of gastrointestinal microbiota in fish. *Aquac Res* **41,** 1553-1573.

Newman, M., Lyons, T. P. and Jacques, K. A. Effects of mannan oligosaccharide source and structure on antibiotic resistance of pathogenic bacteria. Nutritional biotechnology in the feed and food industries: Proceedings of Alltech' s 22nd Annual Symposium, 23-26 April 2006 2006 Lexington, Kentucky, USA. 109-113.

O'Hara, A. M. and Shanahan, F. (2006) The gut flora as a forgotten organ. *EMBO Rep* **7,** 688-693.

Olsen, R., Sundell, K., Hansen, T., Hemre, G.-I., Myklebust, R., Mayhew, T. and Ringø, E. (2002) Acute stress alters the intestinal lining of Atlantic salmon, Salmo salar L.: An electron microscopical study. *Fish Physiol Biochem* **26,** 211-221.

Olsen, R. E., Sundell, K., Mayhew, T. M., Myklebust, R. and Ringø, E. (2005) Acute stress alters intestinal function of rainbow trout, Oncorhynchus mykiss (Walbaum). *Aquaculture* **250,** 480-495.

Pan, X., Wu, T., Zhang, L., Song, Z., Tang, H. and Zhao, Z. (2008) In vitro evaluation on adherence and antimicrobial properties of a candidate probiotic Clostridium butyricum CB2 for farmed fish. *J Appl Microbiol* **105,** 1623-9.

Peres, H., Santos, S. and Oliva-Teles, A. (2014) Blood chemistry profile as indicator of nutritional status in European seabass (Dicentrarchus labrax). *Fish Physiol Biochem* **40,** 1339-1347.

Pond, M. J., Stone, D. M. and Alderman, D. J. (2006) Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (Oncorhynchus mykiss). *Aquaculture* **261,** 194-203.

Ray, A. K., Ghosh, K. and Ringø, E. (2012) Enzyme-producing bacteria isolated from fish gut: a review. *Aquacult Nutr* **18,** 465-492.

Ringø, E. and Song, S. K. (2016) Application of dietary supplements (synbiotics and probiotics in combination with plant products and β-glucans) in aquaculture. *Aquacult Nutr* **22,** 4-24.

Ringø, E., Zhou, Z., Vecino, J. L. G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R. E., Dimitroglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H. L., Martinsen, L. L., De Schryver, P., Bossier, P., Sperstad, S. and Merrifield, D. L. (2016) Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquacult Nutr* **22,** 219-282.

Robles, R., Lozano, A. B., Sevilla, A., Márquez, L., Nuez-Ortín, W. and Moyano, F. J. (2013) Effect of partially protected butyrate used as feed additive on growth and intestinal metabolism in sea bream (Sparus aurata). *Fish Physiol Biochem* **39,** 1567-1580.

Romero, J., Ringø, E. and Merrifield, D. L. (2014) The Gut Microbiota of Fish. In Aquacult Nutr, ed. *75-100.* John Wiley & Sons, Ltd.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. and Weber, C. F. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75,** 7537-41.

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A. and Rawls, J. F. (2012) Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host Microbe* **12,** 277-88.

Spring, P. (2003) Intestinal microflora and the possibility to influence it with mannan oligosaccharide. *Prax Vet* **51,** 25-35.

Spring, P., Wenk, C., Dawson, K. A. and Newman, K. E. (2000) The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. *Poultry Sci* **79,** 205-211.

Sullam, K. E., Essinger, S. D., Lozupone, C. A., O'Connor, M. P., Rosen, G. L., Knight, R., Kilham, S. S. and Russell, J. A. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Mol Ecol* **21,** 3363-78.

Torrecillas, S., Makol, A., Benitez-Santana, T., Caballero, M. J., Montero, D., Sweetman, J. and Izquierdo, M. (2011) Reduced gut bacterial translocation in European sea bass (Dicentrarchus labrax) fed mannan oligosaccharides (MOS). *Fish Shellfish Immunol* **30,** 674-81.

Vine, N. G., Leukes, W. D., Kaiser, H., Daya, S., Baxter, J. and Hecht, T. (2004) Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. *J Fish Dis* **27,** 319-26.

Waché, Y., Auffray, F., Gatesoupe, F.-J., Zambonino, J., Gayet, V., Labbé, L. and Quentel, C. (2006) Cross effects of the strain of dietary Saccharomyces cerevisiae and rearing conditions on the onset of intestinal microbiota and digestive enzymes in rainbow trout, Onchorhynchus mykiss, fry. *Aquaculture* **258,** 470-478.

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P. B., Welch, T., Wiens, G. D., Snekvik, K., Rawls, J. F. and Good, C. (2013) Aquacultured rainbow trout (Oncorhynchus mykiss) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Appl Environ Microbiol* **79,** 4974-84.

Zorriehzahra, M. J., Delshad, S. T., Adel, M., Tiwari, R., Karthik, K., Dhama, K. and Lazado, C. C. (2016) Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review. *Vet Quart*, 10.1080/01652176.2016.1172132**,** 1-14.

Figure captions

Figure 1. Alpha diversity of bacterial communities in the intestinal mucosa and contents of juvenile rainbow trout fed functional diets for 30 d. **(A)** Rarefaction curves of intestinal microbial communities from each experimental group; **(B)** Chao1 richness estimator; **(C)** Shannon diversity index and number of OTUs; **(D)** Simpson's diversity index (1-D). Columns represent the average values for each group and black line in C chart represent the number of OTUs for comparison purposes. Error bars indicate standard deviation, and different letters indicate significant differences between diets for each sampling point (Wilcoxon signal rank test; $P < 0.05$).

Figure 2. Principal coordinate analysis score plot indicating the centroids of each experimental group. Phylogenetic relationships are shown between intestinal mucosa and contents in rainbow trout communities after being fed functional diets for 30 d. Beta diversity was calculated for all samples using the UniFrac metric for the V3 and V4 regions of the 16S gene, and principal coordinate analysis was performed based on weighted UniFrac distances. PC1 and PC2 are principal

coordinates 1 and 2, respectively. Symbols are: CTRL-Circles; PRO-Triangles; PRE-Squares; MIX- Crosses

Figure 3. Bacterial phyla found in the intestinal mucosa and contents of rainbow trout fed functional diets for 30 d. Compositions were obtained by sequencing the 16S V3 and V4 regions. Groups were established as follows: CTRL, fish fed only the commercial diet; PRO, probiotic supplemented diet; PRE, prebiotic supplemented diet; and MIX, pre- and probiotic supplemented diet. (Please see online version for color version)

Figure 4. Order-level heatmap for relative OTU abundances (log transformed) from intestinal mucosa and contents of rainbow trout fed functional diets for 30 d. The average relative abundances of OTUs are summarized at the order level for each experimental group at 7, 14, and 30 d. Two-way hierarchical clustering grouped and ordered taxa (rows) and dietary groups (columns)through complete-linkage hierarchical clustering. Groups were established as follows: CTRL, fish fed only the commercial diet; PRO, probiotic supplemented diet; PRE, prebiotic supplemented diet; and MIX, pre- and probiotic supplemented diet. (Please see online version for color version)

Figure 5. **(A)** Venn diagrams representing shared and unique operational taxonomic units (OTUs) identified in rainbow trout intestine mucosa and contents after being fed functional diets for 7, 14, and 30 d. **(B)** Table representing the core microbiome taxa identified in all fish regardless of diet and feeding time.

Supporting Information

Additional File 1: Figure S1. Experimental design

Additional File 2: Figure S2. Classes abundances per group

Additional File 3: Table S1. Summary of the paired-end merging result on the 57 samples of the study by FLASH.

Additional File 4: Table S2. Summary of the results of clustering and preprocessing by CD-HIT-OTU

Additional File 5: Table S3. Summary of all OTUs count in each sample

Additional File 6: Table 4S. Permanova contrast analysis

Table 1. Proximate composition of different experimental functional diets

	Diet			
Ingredients (%)	**CTRL**	**PROBIOTIC**	**PREBIOTIC**	**MIX**
Crude Protein	48	48	48	48
Lipids	16	16	16	16
Humidity	11	11	11	11
Ash	11	11	11	11
Crude fiber	2,5	2,5	2,5	2,5
S. cerevisiae	0	0,5	0	0,5
Mannan-oligosaccharides	0	0	0,6	0,6

Diet proximate composition available from EWOS-Cargill; Mannan-oligosaccharides provided from a commercial source with a minimum of 12% mannan-oligosaccharides in the mixture.

Table 2. Plasma biochemistry levels and condition factor of juvenile rainbow trout fed functional diets for 30 days

	7 d				14 d				30 d				2-Way ANOVA P-value		
Parameter	**CTRL**	**PRO**	**PRE**	**MIX**	**CTRL**	**PRO**	**PRE**	**MIX**	**CTRL**	**PRO**	**PRE**	**MIX**	**Diet**	**Time**	**D x T**
GLU (mg/mL)	86.1± 10.5	86.6 ± 9.6	77.2 ± 9.9	75.2 ± 3.4	78.3 ± 6.6^{a}	75.4 ± 20.3^{ab}	51.2 ± 14.0^{b}	61.0 ± 7.3^{ab}	75.8 ± 21.9^{a}	75.3 ± 11.2^{a}	91.8 ± 13.9^{b}	95.4 ± 15.1^{b}	0.041	<0.001	<0.001
TG (mg/mL)	153.6 ± 16.5^{a}	147.5 ± 21.3^{a}	177.0 ± 44.6^{ab}	186.1 ± 50.0^{b}	169.8 ± 17.5	157.3 ± 33.0	163.9 ± 22.9	184.4 ± 26.7	126.7 ± 36.5^{a}	142.8 ± 27.2^{ab}	149.5 ± 31.2^{ab}	202.5 ± 89.8^{b}	0.027	ns	ns
TCHO (mg/mL)	273.6 ± 63.0	204.4 ± 140.9	244.7 ± 110.1	249.8 ± 31.2	198.8 ± 70.6	139.6 ± 45.6	177.8 ± 75.8	147.0 ± 47.1	112.7 ± 38.6	110.3 ± 22.9	102.4 ± 24.8	113.6 ± 13.4	ns	<0.001	ns
GGT (U/L)	7.5 ± 4.4^{a}	5.1 ± 3.2^{ab}	3.3 ± 3.0^{b}	5.2 ± 3.9^{ab}	9.1 ± 1.6^{a}	8.7 ± 4.8^{a}	3.8 ± 2.7^{b}	3.5 ± 1.2^{b}	6.8 ± 6.3	9.3 ± 4.4	8.9 ± 2.3	7.6 ± 2.7	0.032	ns	ns
AST (U/L)	50.6 ± 23.2	33.5 ± 9.4	37.3 ± 17.1	56.3 ± 31.7	80.9 ± 37.2^{a}	55.3 ± 36.1^{ab}	25.7 ± 17.8^{b}	25.3 ± 18.8^{b}	25.6 ± 12.2	28.3 ± 11.3	28.5 ± 8.4	34.8 ± 16.2	ns	0.037	0.049
ALT (U/L)	1.4 ± 0.9	1.8 ± 1.2	1.9 ± 1.3	4.4 ± 4.7	6.3 ± 3.3^{a}	6.4 ± 5.8^{a}	4.7 ± 2.1^{ab}	3.6 ± 1.7^{b}	5.1 ± 6.5^{a}	3.4 ± 0.9^{a}	0.9 ± 1.2^{b}	2.9 ± 1.0^{a}	ns	0.01	0.047
Fulton's K	1.09 ± 0.11	1.08 ± 0.11	1.15 ± 0.05	1.13 ± 0.06	1.06 ± 0.05	1.10 ± 0.06	1.02 ± 0.04	1.03 ± 0.07	1.06 ± 0.08	1.08 ± 0.04	1.04 ± 0.06	1.12 ± 0.08	ns	ns	ns

Abbreviations: CTRL- group fed control diet; PRO-group fed diet supplemented with probiotic *S. cerevisiae*; PRE-group fed diet supplemented with prebiotic mannanoligosaccharides; MIX- group fed diet supplemented with the mix of both pre and probiotic: GLU- Glucose; TG-Triglycerides; TCHO-Total cholesterol; GGT-Gamma glutamyl transpeptidase; AST- Aspartate transaminase; ALT- Alanine transaminase; ns- not significant. Values are average ± standard deviation and different letters indicate significant differences between the experimental diets on the respective time (n=3/replica; Tukey HSD; P<0.05)

Table 3. Two-way PERMANOVA output

Source	df	Pseudo-F test	P-value
Time	2	5.11	0.012
Diet	3	4.32	0.011
Time x Diet	6	3.21	0.014

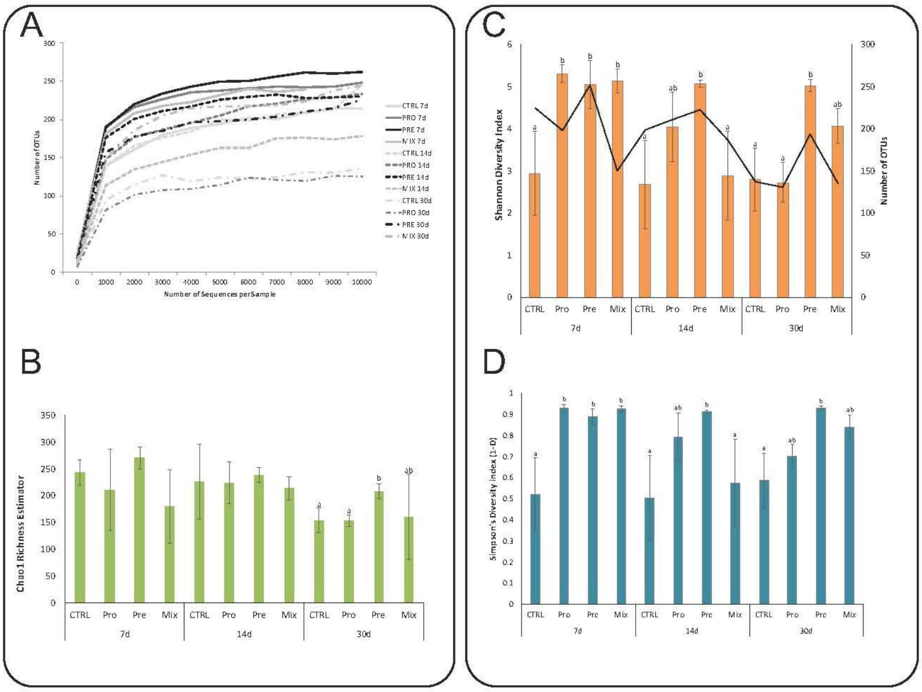


Figure 1

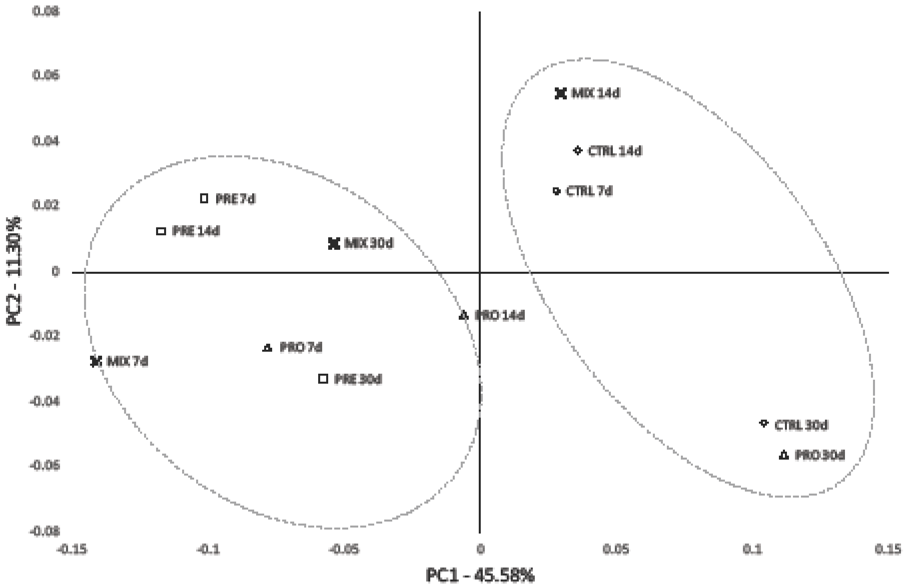

0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
PC2 - 11.30%
-0.15
-0.1
-0.05
0
0.05
0.1
0.15
PC1 - 45.58%
MIX 14d
CTRL 14d
CTRL 7d
PRE 7d
PRE 14d
MIX 30d
PRO 14d
PRO 7d
MIX 7d
PRE 30d
CTRL 30d
PRO 30d

Figure 2

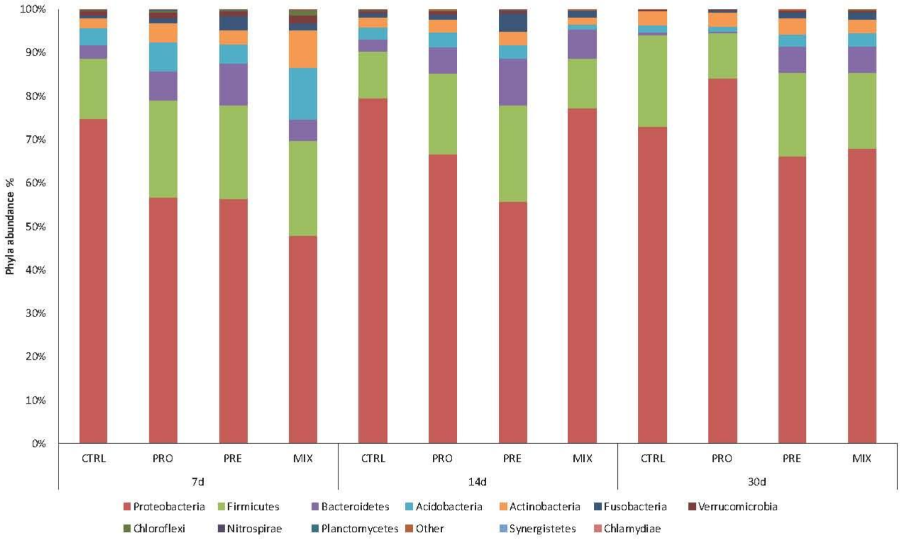

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Phyla abundance %
CTRL
PRO
PRE
MIX
7d
14d
30d
Proteobacteria
Firmicutes
Bacteroidetes
Acidobacteria
Actinobacteria
Fusobacteria
Verrucomicrobia
Chloroflexi
Nitrospirae
Planctomycetes
Other
Synergistetes
Chlamydiae

Figure 3

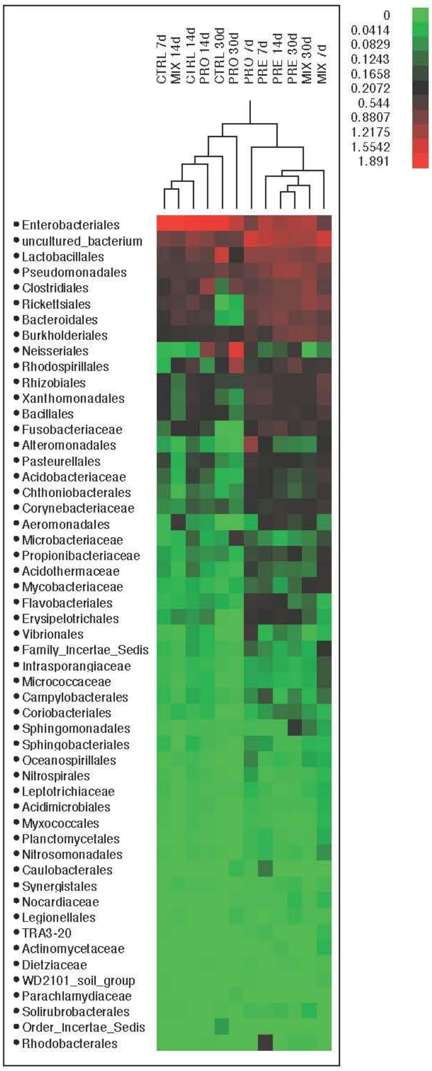
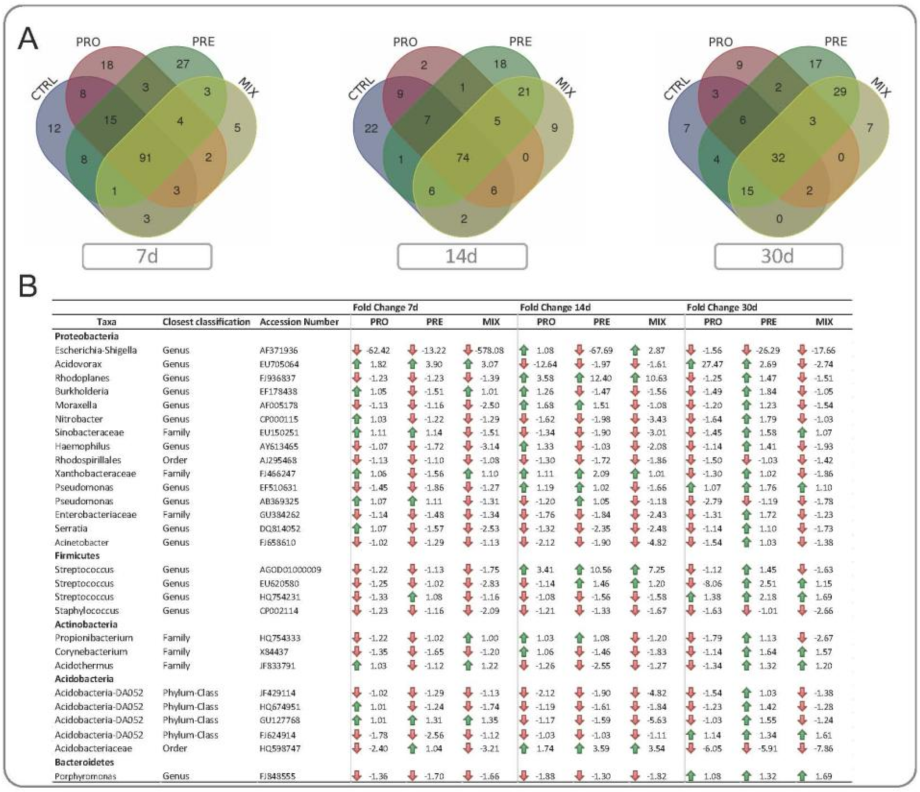

CTRL 7d
MIX 14d
CTRL 14d
PRO 14d
CTRL 30d
PRO 30d
PRO 7d
PRE 7d
PRE 14d
PRE 30d
MIX 30d
MIX 7d
0
0.0414
0.0829
0.1243
0.1658
0.2072
0.544
0.8807
1.2175
1.5542
1.891
Enterobacteriales
uncultured_bacterium
Lactobacillales
Pseudomonadales
Clostridiales
Rickettsiales
Bacteroidales
Burkholderiales
Neisseriales
Rhodospirillales
Rhizobiales
Xanthomonadales
Bacillales
Fusobacteriaceae
Alteromonadales
Pasteurellales
Acidobacteriaceae
Chthoniobacterales
Corynebacteriaceae
Aeromonadales
Microbacteriaceae
Propionibacteriaceae
Acidothermaceae
Mycobacteriaceae
Flavobacteriales
Erysipelotrichales
Vibrionales
Family_Incertae_Sedis
Intrasporangiaceae
Micrococcaceae
Campylobacterales
Coriobacteriales
Sphingomonadales
Sphingobacteriales
Oceanospirillales
Nitrospirales
Leptotrichiaceae
Acidimicrobiales
Myxococcales
Planctomycetales
Nitrosomonadales
Caulobacterales
Synergistales
Nocardiaceae
Legionellales
TRA3-20
Actinomycetaceae
Dietziaceae
WD2101_soil_group
Parachlamydiaceae
Solirubrobacterales
Order_Incertae_Sedis
Rhodobacterales

Figure 4



			Fold Change 7d			Fold Change 14d			Fold Change 30d		
Taxa	**Closest classification**	**Accession Number**	**PRO**	**PRE**	**MIX**	**PRO**	**PRE**	**MIX**	**PRO**	**PRE**	**MIX**
Proteobacteria											
Escherichia-Shigella	Genus	AF371936	↓ -62.42	↓ -13.22	↓ -578.08	↑ 1.08	↓ -67.69	↑ 2.87	↓ -1.56	↓ -26.29	↓ -17.66
Acidovorax	Genus	EU705064	↑ 1.82	↑ 3.90	↑ 3.07	↓ -12.64	↓ -1.97	↓ -1.61	↑ 27.47	↑ 2.69	↓ -2.74
Rhodoplanes	Genus	FJ936837	↓ -1.23	↓ -1.23	↓ -1.39	↑ 3.58	↑ 12.40	↑ 10.63	↓ -1.25	↑ 1.47	↓ -1.51
Burkholderia	Genus	EF178438	↑ 1.05	↓ -1.51	↑ 1.01	↑ 1.26	↓ -1.47	↓ -1.56	↓ -1.49	↑ 1.84	↓ -1.05
Moraxella	Genus	AF005178	↓ -1.13	↓ -1.16	↓ -2.50	↑ 1.68	↑ 1.51	↓ -1.08	↓ -1.20	↑ 1.23	↓ -1.54
Nitrobacter	Genus	CP000115	↑ 1.03	↓ -1.22	↓ -1.29	↓ -1.62	↓ -1.98	↓ -3.43	↓ -1.64	↑ 1.79	↓ -1.03
Sinobacteraceae	Family	EU150251	↑ 1.11	↑ 1.14	↓ -1.51	↓ -1.34	↓ -1.90	↓ -3.01	↓ -1.45	↑ 1.58	↑ 1.07
Haemophilus	Genus	AY613465	↓ -1.07	↓ -1.72	↓ -3.14	↑ 1.33	↓ -1.03	↓ -2.08	↓ -1.14	↑ 1.41	↓ -1.93
Rhodospirillales	Order	AJ295468	↓ -1.13	↓ -1.10	↓ -1.08	↓ -1.30	↓ -1.72	↓ -1.86	↓ -1.50	↓ -1.03	↓ -1.42
Xanthobacteraceae	Family	FJ466247	↑ 1.06	↓ -1.56	↑ 1.10	↑ 1.11	↑ 2.09	↑ 1.01	↓ -1.30	↑ 1.02	↓ -1.86
Pseudomonas	Genus	EF510631	↓ -1.45	↓ -1.86	↓ -1.27	↑ 1.19	↑ 1.02	↓ -1.66	↑ 1.07	↑ 1.76	↑ 1.10
Pseudomonas	Genus	AB369325	↑ 1.07	↑ 1.11	↓ -1.31	↓ -1.20	↑ 1.05	↓ -1.18	↓ -2.79	↓ -1.19	↓ -1.78
Enterobacteriaceae	Family	GU384262	↓ -1.14	↓ -1.48	↓ -1.34	↓ -1.76	↓ -1.84	↓ -2.43	↓ -1.31	↑ 1.72	↓ -1.23
Serratia	Genus	DQ814052	↑ 1.07	↓ -1.57	↓ -2.53	↓ -1.32	↓ -2.35	↓ -2.48	↓ -1.14	↑ 1.10	↓ -1.73
Acinetobacter	Genus	FJ658610	↓ -1.02	↓ -1.29	↓ -1.13	↓ -2.12	↓ -1.90	↓ -4.82	↓ -1.54	↑ 1.03	↓ -1.38
Firmicutes											
Streptococcus	Genus	AGOD01000009	↓ -1.22	↓ -1.13	↓ -1.75	↑ 3.41	↑ 10.56	↑ 7.25	↓ -1.12	↑ 1.45	↓ -1.63
Streptococcus	Genus	EU620580	↓ -1.25	↓ -1.02	↓ -2.83	↓ -1.14	↑ 1.46	↑ 1.20	↓ -8.06	↑ 2.51	↑ 1.15
Streptococcus	Genus	HQ754231	↓ -1.33	↑ 1.08	↓ -1.16	↓ -1.08	↓ -1.56	↓ -1.58	↑ 1.38	↑ 2.18	↑ 1.69
Staphylococcus	Genus	CP002114	↓ -1.23	↓ -1.16	↓ -2.09	↓ -1.21	↓ -1.33	↓ -1.67	↓ -1.63	↓ -1.01	↓ -2.66
Actinobacteria											
Propionibacterium	Family	HQ754333	↓ -1.22	↓ -1.02	↑ 1.00	↑ 1.03	↑ 1.08	↓ -1.20	↓ -1.79	↑ 1.13	↓ -2.67
Corynebacterium	Family	X84437	↓ -1.35	↓ -1.65	↓ -1.20	↑ 1.06	↓ -1.46	↓ -1.83	↓ -1.14	↑ 1.64	↑ 1.57
Acidothermus	Family	JF833791	↑ 1.03	↓ -1.12	↑ 1.22	↓ -1.26	↓ -2.55	↓ -1.27	↓ -1.34	↑ 1.32	↑ 1.20
Acidobacteria											
Acidobacteria-DA052	Phylum-Class	JF429114	↓ -1.02	↓ -1.29	↓ -1.13	↓ -2.12	↓ -1.90	↓ -4.82	↓ -1.54	↑ 1.03	↓ -1.38
Acidobacteria-DA052	Phylum-Class	HQ674951	↑ 1.01	↓ -1.24	↓ -1.74	↓ -1.19	↓ -1.61	↓ -1.84	↓ -1.23	↑ 1.42	↓ -1.28
Acidobacteria-DA052	Phylum-Class	GU127768	↑ 1.01	↑ 1.31	↑ 1.35	↓ -1.17	↓ -1.59	↓ -5.63	↓ -1.03	↑ 1.55	↓ -1.24
Acidobacteria-DA052	Phylum-Class	FJ624914	↓ -1.78	↓ -2.56	↓ -1.12	↓ -1.03	↓ -1.03	↓ -1.11	↑ 1.14	↑ 1.34	↑ 1.61
Acidobacteriaceae	Order	HQ598747	↓ -2.40	↑ 1.04	↓ -3.21	↑ 1.74	↑ 3.59	↑ 3.54	↓ -6.05	↓ -5.91	↓ -7.86
Bacteroidetes											
Porphyromonas	Genus	FJ848555	↓ -1.36	↓ -1.70	↓ -1.66	↓ -1.88	↓ -1.30	↓ -1.82	↑ 1.08	↑ 1.32	↑ 1.69

Figure 5