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Regulation of Host Gene Expression by Gastrointestinal Tract Microbiota in Chinook Salmon (*Oncorhynchus tshawytscha*)

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Abstract

Differences in gut microbiome composition are linked with health, disease and ultimately host fitness; however, the molecular mechanisms underlying that relationship are not well characterized. Here, we modified the fish gut microbiota using antibiotic and probiotic feed treatments to address the effect of host microbiome on gene expression patterns. Chinook salmon (*Oncorhynchus tshawytscha*) gut gene expression was evaluated using whole transcriptome sequencing (RNA-Seq) on hindgut mucosa samples from individuals treated with antibiotic, probiotic and control diets to determine differentially expressed (DE) host genes. Fifty DE host genes were selected for further characterization using nanofluidic qPCR chips. We used 16S rRNA gene metabarcoding to characterize the rearing water and host gut microbiome (bacterial) communities. Daily administration of antibiotics and probiotics resulted in significant changes in fish gut and aquatic microbiota as well as more than 100 DE genes in the antibiotic and probiotic treatment fish, relative to healthy controls. Normal microbiota depletion by antibiotics mostly led to downregulation of different aspects of immunity and upregulation of apoptotic process. In the probiotic treatment, genes related to post-translation modification and inflammatory responses were up-regulated relative to controls. Our qPCR results revealed significant effects of treatment (antibiotic and probiotic) on *rabep2, aifm3, manf, prmt3* gene transcription. Moreover, we found significant associations between members of *Lactobacillaceae* and *Bifidobacteriaceae* with host gene expression patterns. Overall, our analysis showed that the microbiota had significant impacts on many host signaling pathways, specifically targeting immune, developmental, and metabolic processes. Our characterization of some of the molecular mechanisms involved in microbiome-host interactions will help develop new strategies for preventing/ treating microbiome disruption-related diseases.

Keywords: Probiotics, Antibiotics, Transcriptome, Host-microbe interactions, Fish microbiome

Introduction

Nearly all animals examined to date show complex interactions with their associated microbial communities. It is evident that there are bidirectional interactions between the gut microbiome and the host in humans (Davison et al., 2017; Dayama et al., 2020; Meisel et al., 2018) and non-human animals (Fuess et al., 2021; Muehlbauer et al., 2021; Naya-Catala et al., 2021). These interactions affect a wide range of host phenotypes including metabolism, immunity, and physiology (McFall-Ngai et al., 2013). Recent studies have shown that host genetics can also shape their gut microbiome (Lopera-Maya et al., 2022; Piazzon et al., 2020). The evidence for benefits provided by the gut microbiota is growing for example, gut microbiota can improve nutrition absorption (Krajmalnik-Brown et al., 2012), facilitate resistance against pathogens (Ducarmon et al., 2019), train the immune system and even modify behaviour and mental state (Surana and Kasper, 2017). Moreover, the gut microbiota gain substantial benefits from their host (e.g., available nutrients and suitable habitat) resulting in a mutualistic relationship with the host. This provides the context for a unique coevolved process in which host and their gut microbiome interact in a mutualistic adaptive scenario (Minich et al., 2022; Escalas et al., 2021; Groussin et al., 2020). Coevolution is defined as the reciprocal adaptation process experienced by two organisms as the result of their reciprocal selection pressures; it is possible for the microbiome to evolve at the individual species level, as well as a community response to host-mediated selection (Koskella and Bergelson, 2020).

Many studies have shown the importance of the gut microbiome in healthy and diseased host states, which ultimately affects host fitness (Bozzi et al., 2021; Manor et al., 2020; Yao et al., 2018). The gut microbiome has been shown to alter host gene expression (Davison et al., 2017; Nichols and Davenport, 2021), perhaps a mechanism for the effect of the microbiome on the host. However, the mechanisms and direction of these effects is still not clear since the evidence is largely correlational. Does a change in microbiome composition cause changes in host gene expression, and if so, which genes will be most impacted? It is clearly important to characterize the mechanisms through which the microbiome can cause changes in host gene expression.

Fish live in diverse aquatic environments, but they all harbour complex and diverse microbiomes, and those microbial communities start developing when the eggs are laid (Llewellyn et al., 2014). The bidirectional interaction between the host gut and its associated microbes may arguably be better established in fish, relative to terrestrial animals, as fish are in

constant direct contact with the aquatic environmental microbiome through their gut, gills, and skin. Moreover, given the long evolutionary history of fish as a group, studying host–microbe co-evolution in fish may provide unique insights into the host–microbe relationships in general (Montalban-Arques et al., 2015). Characterizing the mechanisms of how the gut microbiota and gene expression processes of the host interact in a symbiotic manner will help explain the physiological processes that maintain the balance among these intricate cross-kingdom interactions and ultimately, help attempts to prevent dysbiosis (Nichols and Davenport, 2021). Most studies on host- microbiome interactions are correlative or associative analyses without clearly defined cause and effect (Surana and Kasper, 2017). To move beyond such studies, we must more directly address causation through perturbation experimental analyses (Xia and Sun, 2017). Using probiotics and antibiotics to alter gut microbiome (bacterial communities) in healthy hosts can provide valuable experimental insight into the mechanisms of host-microbiome interactions. Antibiotics can be used for antibiotic-induced microbiome depletion (AIMD), this leads to changes in the structure and function of the gut microbial communities (Ferrer et al., 2017). Furthermore, probiotics can also be used to alter the gut microbiome in a controlled manner, as well as stimulate the host intestinal immune system (Lee and Bak, 2011). Experimental perturbations of the gut microbial community with probiotic strains in human and animal disease treatment is well documented (Azad et al., 2018). However, the effect of probiotics in healthy individuals is not as well characterized.

The direction and nature of host-gut microbiome interactions is still an open question in the study of the microbiome, although it is likely bidirectional and experimental analyses of the mechanisms behind these interactions are needed. Here, our goal was to explore a broad range of host gut tissue responses induced by the experimental manipulation of the gut microbiome. We chose Chinook salmon (*Oncorhynchus tshawytscha*) as our study organism as they are reared for commercial and conservation purposes and provide logistical advantages for a study such as ours. Specifically, we used antibiotic, probiotic and control diet treatments to manipulate the gut microbiome in families of Chinook salmon. We used 16S rRNA metabarcoding of the gut bacterial community, coupled with host gut tissue transcriptomics to; (i) quantify treatment effects on the host gut and the fish rearing water bacterial community compositions, (ii) determine the response of the host gut tissue transcriptome to the treatments, and (iii) use gene transcriptional profiling Taqman^{TM} qPCR to characterize the host response to the treatment-altered gut microbiome. Given the long evolutionary history of the relationship between fish and

their microbiomes, we expect strong bidirectional effects, but predicted that the effects of the microbiome on the host are more pronounced. We specifically hypothesized that the host transcriptional responses to each treatment could be attributed to the abundance of specific bacterial taxa. The results obtained provide insight into the co-evolved symbiotic relationship between host and its associated microbiome that may inform future studies exploring host-microbiome interactions and evolution. Additionally, our work will help in better using microbiome manipulation (probiotics, antibiotics) to improve health in fishes and potentially in other animals, including humans.

Materials and methods

Study design

We domesticated Chinook salmon from Yellow Island Aquaculture Ltd, an organic salmon farm on Quadra Island, BC, Canada to create a nested breeding design with two sires crossed with one dam (2×1) replicated six times. Eggs were fertilized in October 2019 and the eggs were incubated in replicate cells of vertical stack incubation trays. When the offspring reached the first feeding (March 2020) offspring from replicated incubation tray cells were transferred to 200 L tanks with well water flow of 2 L per minute with continuous aeration with a 16:8 h light-dark cycle. Fish were fed ~3% of their body weight three times per day until October 24th, 2020. At that time, 5 fish per family were moved to new 200 L tanks for a total of 72 tanks (12 (families)*2 (replicates)* 3 (treatments – see below)).

Microbiome manipulation

We manipulated the gut microbiome of the fish in the tanks using control (untreated) feed, antibiotic treated feed and probiotic treated feed:

Antibiotic treatment: Oxytetracycline (OTC), and Chloramphenicol (CAP), two broad spectrum antibiotics, were selected for the trial. Twenty-four tanks (for 12 (families)*2 (replicates)) were labelled as antibiotic and were treated with OTC (83 mg/kg/day concentration) (Kokou et al., 2020; Rosado et al., 2019, Leal et al., 2019) for six days. After six days the fish were switched to a combination of Chloramphenicol (CAP) (42 mg/kg/day) (Bilandzic et al., 2012) plus the OTC for four more days, for a total of 10 days of antibiotic treatment. Fish were fed three times a day at approximately 3% of their body weight.

Probiotic treatment: Twenty-four tanks (for 12 (families)*2 (replicates)) were labelled as probiotic treatment and fed commercially available Jamieson Probiotic Complex with 60 billion colony forming units (CFU) (Jamieson Laboratories, Canada; Supplementary Table S1). Specifically, the probiotic-treated feed (3 capsules per 100 gram of feed) was coated with 10 mL of sodium alginate (1%) and 10 mL of 0.5 % calcium chloride prior to mixing with the probiotic powder. Fish were fed three times a day at approximately 3% of their body weight.

Control: Twenty-four tanks (for 12 (families)*2 (replicates)) were labelled as control group and fish were fed with regular feed without probiotic or antibiotic for ten days. Fish were fed three times a day at approximately 3% of their body weight (Fig. 1).

Sampling

All fish were terminally sampled after the ten-day trial (November 3, 2020). The fish were not fed on the day of sampling. The final mean mass of the fish was 23.3 g (±7.2 SE) across all families and treatments (no treatment effect on fish body weight was detected). Three fish were dip netted from each tank and humanely euthanized immediately in an overdose solution of clove oil (Toews et al., 2019). Of the 72 tanks, four tanks (control) had 100 % mortality and those replicates were excluded from the study, bringing the total number of samples to 204 fish (72 probiotic treated fish, 72 antibiotic treated fish, 60 control fish) and 68 water samples (one per tank)). The sampled fish were immediately weighed and dissected, with the entire GI tract placed in a 50 mL tube with 35 mL of a highly concentrated salt buffer (ammonium sulfate, 1 M sodium citrate, 0.5 M EDTA, H_2SO_4 to bring the pH to 5.2) for preservation for later RNA and DNA extraction. Additionally, 500 mL water samples were collected from each of the tanks (N=68) before sampling the fish and filtered immediately using 0.22-micron pore size, 47 mm diameter polycarbonate filters (Isopore™, Millipore, MA). All samples (tissue in preservative and the filters) were stored at –20 °C, until used for DNA or RNA extraction.

Lab Analyses

The lab analyses consisted of three related but separate protocols (Figure 1). The first was to assess the bacterial composition of the fish gut and rearing water microbiomes using 16S rRNA metabarcoding. The second was to determine the whole transcriptome response to treatment by RNA-Seq of gut tissue from offspring form a single family. The third analysis was designed to better characterize the transcriptional profile response to the treatments using nanofluidic array qPCR analysis of 50 gene loci selected using the RNA-Seq analysis.

Bacterial DNA extraction and 16S rRNA gene library preparation

DNA was extracted from fish hindgut content using a sucrose lysis buffer solution method previously described (Shahraki et al., 2019) and extracted DNA was subsequently stored at –20 °C, until further analysis. Additionally, the PCR conditions and 16S rRNA primer sets (1st and second PCR) were the same as those used in previously described methods (Sadeghi et al., 2021). Briefly, the V5 (787 F-acctgcctgccg-ATTAGATACCCNGGTAG) and V6 (1046 R-acgccaccgagc-CGACAGCCATGCANCACCT) variable regions of the 16S rRNA were selected for PCR amplification with a PCR cycle program of 95 °C for 3 min followed by 28 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 m, and a final step at 72 °C for 7 m. A second short-cycle PCR (7 cycles) using purified first PCR products ligated the adaptor and barcode (10 -12 bp) sequences to the amplicons as required for sample identification and sequencing. During the

first and second PCR, nine samples failed amplification and 263 samples (195 gut samples, and 68 water samples) remained for the gel extraction. For each 96 well PCR plate, one negative control consisting of PCR mix (of first and second PCR) with ultra-pure water instead of DNA template was included. The pooled purified PCR amplicon mix (i.e., sequencing library) was sequenced on an ION S5 Next-Generation Sequencing system.

16S Metabarcode Sequence Data Processing

The resulting FASTQ file was analyzed using the Quantitative Insights Into Microbial Ecology (QIIME2-2020.11) platform (Bolyen et al., 2019). The FASTQ sequence file was demultiplexed and the DADA2 pipeline was used to denoise single-end sequences, dereplicate and filter chimeras. This was followed by Amplicon Sequence Variant (ASV) picking using the removeBimeraDenovo function with the "consensus" method, while default values were used for the other parameters (Callahan et al., 2016). Taxonomic classification was done through the feature-classifier plugin (Bokulich et al., 2018) using the SILVA 138-99 reference database (Quast et al., 2013). This plugin supports taxonomic classification of features using the Naive Bayes method. All ASVs were aligned with mafft (Katoh et al., 2002) and used to construct a phylogeny with fasttree (Price et al., 2010). A total of 8,820,568 sequences with 19,776 ASVs were obtained for the 267 samples (195 gut samples, 68 water samples, and 4 negative controls). The four negative controls had 1 to 7 reads and were excluded from the rest of the study. Using a taxon filter-table, ASVs related to eukaryotes, mitochondria, chloroplasts (combined ~ 1%), and unassigned (1%), were removed, resulting in a total of 8,655,659 (98%) sequences remaining. Furthermore, samples with low sequence depth (less than 3000 reads), low abundance taxa (less than 10 ASVs) and ASVs that showed up in only one sample were removed. This decreased the total number of samples to 255 samples (189 gut samples, 66 water samples) with 8,217,478 sequences and 2888 ASVs. The 8 deleted samples were not related to specific treatment type or family (antibiotic treatment (one water sample), probiotic treatment (4 gut samples, and one water sample), control (two gut samples)). Alpha diversity indices (Chao1 (a metric for species richness), and Faith's phylogenetic diversity (PD) (a metric that incorporates both species richness and species evenness while correcting for phylogenetic distance)) of bacterial communities were calculated using the QIIME2 alpha diversity plugin. The ASV table was rarefied to 3000 reads per sample for the alpha diversity estimation (rarefaction curves plateaued at 3000 reads). Bray–Curtis, and Jaccard dissimilarity distance matrixes were calculated to estimate β-diversity.

RNA extraction

RNA was extracted from host hindgut tissue using TRIzol® reagent (Life Technologies, Mississauga, ON, CAT=15596018) following the manufacturer's protocol. RNA was dissolved in sterile water and treated with TURBO™ DNase (Life Technologies, Mississauga, ON) to remove genomic DNA contamination and preserved at –80°C until RNA sequencing or cDNA synthesis and qPCR were performed (see below).

RNA sequencing and transcriptome assembly

A total of 18 gut tissue samples from one family, but from all 3 treatments (6 fish per treatment), were used for transcriptome analyses by RNAseq. Fish from one family were used to minimize differences due to genetic variability among individuals. RNA quality was assessed using the Eukaryotic RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent, Mississauga, ON). All samples had an RIN > 7 and a 28S:18S rRNA ratio >1.0. RNAseq libraries were prepared and sequenced at the McGill University and Genome Quebec Innovation Centre using the Illumina NovaSeq 6000 S4 PE100 protocol and 100-bp paired-end sequencing. To remove potentially contaminating rRNA sequences, raw sequences were filtered against eight default rRNA databases using SortMeRNA v2.1 (Kopylova et al., 2012). The sequences were then quality-filtered using Trimmomatic v0.38 (Bolger et al., 2014). The non-rRNA sequences were aligned to the Chinook salmon (GCF_002872995.1_Otsh_v1.0; https://www.ncbi.nlm.nih.gov/assembly/GCF_002872995.1/) reference genome using the splicing aligner HISAT2 (Kim et al., 2015). FeatureCounts (Liao et al., 2014), was used to calculate the number of transcript sequence fragments assigned to each gene.

Differential expression gene analysis

The output from FeatureCounts was imported into DESeq2 (version '1.32.0') (Love et al., 2014) in R (R version 4.1.1) (Team, 2013) for normalization and differentially expressed genes analysis.

qPCR Primer/probe optimization and cDNA synthesis

Primer and probe optimization: Fifty transcripts (genes) that were significantly DE between antibiotic and probiotic treatments versus the control treatment in the DESeq2 analysis were selected for printing on OpenArray Taqman qPCR chips (Supplementary Table S2). Four endogenous control genes (β-2-microglobulin, β-Actin, ribosomal protein L13, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were selected from previous studies (Geffroy et al., 2021; Limbu et al., 2018; Toews et al., 2019) to normalise the transcription

profiles of the candidate transcripts Primers for the candidate transcripts were designed using Geneious Software v7.1.5 (http://www.geneious.com) and optimized on DNA from Chinook salmon fry. After PCR optimization, the primers were tested on a subset of our cDNA samples with SyBr® Green Dye I (Thermo Fisher Scientific) following the manufacturer's protocol on the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). After testing positive for amplification of the expected sized fragment using SyBr® Green assays, new qPCR primers and Taqman® probes were developed using Primer Express® Software v3.0.1 (Thermo Fisher Scientific) for all 54 genes (50 candidate and 4 control genes; Supplementary Table 1). The qPCR primers spanned intron-exon boundaries with a short amplicon size (50–100 bp). The Taqman® probe was designed for a melting temperature between 57 and 60 °C.

cDNA synthesis: RNA was quality tested on a random subset of the samples both on a 2100 Bioanalyzer and on 2% agarose gels. RNA Integrity Number (RIN) values were consistent among samples, ranging between 7 and 9.8, while gel images showed the expected rRNA bands. The RNA concentration for each sample was estimated by Spark® multimode microplate reader and NanoQuant Plate™ (Tecan, Morrisville, NC, USA). All total RNA preparations had purity values of 1.8 – 2.1 (A260/A280) with concentrations ranging from 2,000 to 5,000 ng/µL. TURBO DNA-free™ Kits (Thermo Fisher Scientific, cat. no. AM1907) were used to remove genomic DNA contamination. Total RNA was converted to cDNA using High Capacity cDNA Kits (Applied Biosystems, Ontario, Canada), following the manufacturer's protocol. Reverse transcriptase reactions contained 10 µL of total RNA at a concentration of 200 ng/µL, 2 µL of 10X RT random primers (Applied Biosystems), 0.8 µL of dNTP (100mM), 50 U of MultiScribe RT (Applied Biosystems) and 40 U of RNase Inhibitor (Applied Biosystems) in a 2 µL of 10X RT buffer at a final volume of 20 µL. RT reactions were incubated at 25°C for 10 min followed by 37°C for 120 min and were stopped by incubating at 85°C for 5 min. cDNA samples were stored at –20°C until further analysis.

OpenArray high-throughput qPCR

TaqMan® OpenArray® chips from Applied Biosystems (Burlington, ON, Canada) were used to quantify transcription at the 54 genes (50 candidate and 4 endogenous control genes) on a QuantStudio 12K Flex Real-Time PCR System following the manufacturer's protocol. Forty-eight cDNA samples were run (two chips for 48 samples) for each of the 54 genes on each chip. A 5 µL reaction volume which includes 1.2 µL of cDNA (100ng/µL/per sample), 1.3 µL of ddH_2O and 2.5 µL of TaqMan® OpenArray® Real-Time PCR Master Mix (Applied Biosystems,

Burlington, ON, Canada) was used, aliquoted across a 384-well plate and then loaded onto the TaqMan® OpenArray® chips using the OpenArray® AccuFill System. A total of 10 chips were used for 213 cDNA samples. The samples were randomly distributed among the chips. ExpressionSuite Software (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA) was used to analyse the endogenous control genes. Of four endogenous control genes, β-Actin was selected for normalization due to lower among-sample variation compared to the three other endogenous control genes. Subsequently, all 10 chips were normalized with the selected endogenous control gene (β-Actin) together in ExpressionSuite Software v1.0.3 (Applied Biosystems, Burlington, Ontario, Canada). Moreover, ExpressionSuite Software was used to calculate raw critical threshold (C_T) values and the relative critical threshold values (ΔC_T). Values produced by this platform are already corrected for the efficiency of the amplification (Molina-Lopez et al., 2020). We tested for replicate effect using Paired sample T test in SPSS (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). As we found no evidence for a replicate effect (*P* value > 0.05), C_T and ΔC_T values were averaged between the replicate and only one C_T or ΔC_T value was used for each gene.

Statistical analysis

Treatment effects on bacterial community **composition**

Aquatic bacterial community composition: To test for the effect of treatment on the bacterial community composition in the hold tank water, taxonomical compositions of the bacterial communities were visualized using stacked barplots and Pie charts of the relative abundance of the bacteria at the phylum and family level using the online tool MicrobiomeAnalyst (Chong et al., 2020) as well as R packages ("microbiome" and "phyloseq"). Moreover, differences in alpha diversity indices (Chao1 and PD) among the treatments (antibiotic, probiotic, control) for the tank water bacterial communities were tested using a Kruskal-Wallis (KW) rank test. In the case of a significant association, a *post hoc* Dunn tests with Bonferroni corrected *P* values was done. To visualize among-treatment divergence in the tank water bacterial communities, a Principal-coordinate analysis (PCoA) using two measures of community dissimilarity (Bray–Curtis, and Jaccard) were created. Thus, the significance of the observed clusters was assessed using permutational multivariate analysis of variance (PERMANOVA) and permutational analysis of multivariate dispersions (PERMDISP) in Primer 6 (v6.1.15) as well as QIIME2 (*qiime diversity beta-group-significance*). Pairwise comparisons were performed in cases of significant PERMANOVA among treatment groups.

Fish gut bacterial community composition: The effect of treatment on taxonomic composition of the gut sample bacterial communities was visualized using Pie charts and stacked barplots of the relative abundance of the bacterial taxa at the family and phylum level (Chong et al., 2020). To identify treatment and parental effects on gut microbial community, alpha (Chao1 and PD) diversity indices for gut samples were compared using the KW rank test in SPSS (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). To visualize treatment effects on bacterial community structure, a PCoA using the Bray-Curtis distance matrix was used to generate scatterplot of the first two PCoA axes in R (version 4.1.1). Moreover, PERMDISP and PERMANOVA analyses were performed in R (version 4.1.1) to test for treatment and parental (dams, sires) effects on bacterial community composition. Additionally, QIIME2 was used for creating a PCoA plot as well as PERMANOVA analysis using the Jaccard distance matrix. Pairwise comparisons were performed when significant differences among the treatment groups were detected to identify specific treatment effects.

Comparison between fish gut and aquatic bacterial community composition: Fish gut bacterial community composition was compared against the rearing water bacterial community at both the alpha and beta diversity level. Alpha diversity measures (Chao1 and PD) of gut and water samples were compared using Mann-Whitney U test in SPSS (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp). PCoA first and second axes were used to visualize clustering of the samples based on sample type (gut or water) based on both Jaccard and Bray-Curtis distance matrixes. Subsequently, PERMDISP and PERMANOVA analyses were performed in R (version 4.1.1) to test sample type effect on bacterial community composition.

Gut transcriptome response to treatment

The DESeq2 (version '1.32.0') package in R (version 4.1.1) was used to identify differentially expressed transcripts in the host gut transcriptome between any of the treatment groups in three pairwise comparisons (antibiotic vs control, probiotic vs control, antibiotic vs probiotic). The package uses a Wald test to test the significance of gene transcription differences. To identify differentially expressed transcripts, Benjamini–Hochberg corrections for multiple testing was used (false discovery rate (FDA) < 0.05). We identified differentially expressed transcripts as those genes with thresholds of FDR < 0.05 and $|\log_2 FC| > 1$. Volcano plots of differentially expressed genes between the treatments were generated by using the FC and the log-scaled adjusted *P* value using the EnhancedVolcano package (Blighe et al., 2021) in R.

Transcriptional profile (qPCR) response to treatment

The 50 selected candidate transcripts (hereafter "genes") were tested to determine which genes showed a transcription response to either of the treatments. Two genes (*cfap58*, *ubr4*) were dropped from the analysis due to failure of PCR amplification for most of the samples, thus 48 candidate genes were included for the rest of the study. To reduce the number of independent variables and to avoid over fitting the models, we used Principal Component Analyses (PCA) on the qPCR data for the 48 selected genes using "prcomp" (which is a part of the R statistical analysis package) and factoextra package (1.0.7) (Kassambara and Mundt, 2017) in R (version 4.1.1). Based on a threshold of Eigenvalue > 1, and % variance explained > 2%, the first nine PC axes were selected. We used Linear mixed models (LMM) (lmerTest package (v3.1.3)) (Kuznetsova et al., 2017) in R with the selected PC axes to test for the effect of treatment (fixed effect), and the random effects of dam, sire, fish body weight, tank ID and chip effect, with all interaction terms for fixed and random factors on gene transcription patterns. Chip ID, body weight, dam, treatment×dam, treatment×sire effects were nonsignificant before FDR correction and were removed from the model. When any of the nine PCs were found to exhibit significant effects with any of the independent variables (treatments, dam, sire, body weight, tank ID, or chip effect), we examined the individual gene transcription loading values. We used *fviz_contrib* within the factoextra package (1.0.7) to identify genes with contributions to the PC greater than expected (Kassambara and Mundt, 2017). The identified genes were included in a second analysis that used LMM with the ΔC_T values for the selected genes and the same independent variables (treatment, dam, sire, body weight, tank ID and chip effect), including all interaction terms for fixed and random factors. Nonsignificant factors (Chip ID, body weight, dam, and all interactions) were removed from the model and the analysis was re-run. Lastly, a sequential Bonferroni *P* value correction was applied for multiple testing correction (Rice, 1989).

Correlation between gut bacterial community and host transcriptional profile

To investigate the direct effect of variation in the gut microbiome composition on host gene expression patterns, Spearman's rank correlation coefficient (Spearman's rho) was performed using the function *cor.test* in R (R version 4.2.3). We selected common bacterial taxa (bacteria families with more than 5% contribution to total sequence reads counts within each treatment; (7 taxonomic families) and individual genes with evidence for possible treatment effects (*P* value <0.1 (9 genes)) from the gene-level analysis described above. Moreover, a Holm-Bonferroni (sequential) *P* value correction was applied for multiple testing correction (Rice, 1989). We

visualized the pattern of correlation across all genes and bacterial taxa using a heatmap generated in the pheatmap function in R.

Results

Impact of antibiotics and probiotics on aquatic and fish microbiome.

Microbial community associated with water: We characterized the rearing tank water bacterial communities at two taxonomic levels; the phylum and family. Tank bacterial community diversity diverged among the treatments, with the top 10 most abundant families making up the majority of reads. Proteobacteria were the most common phylum among all treatments (control (70%), antibiotic (68%), probiotic (51%)). Bacteroidota (13 %), and Actinobacteriota (17%) were also common phylum in the control treatment water. Moreover, in the antibiotic treated water, Firmicutes (24%) and Bacteroidota (12%) were common phyla after Proteobacteria. On the other hand, in the probiotic treated water, Bacteroidota (12%) and Firmicutes (8%) were the common phyla after Proteobacteria (Supplementary Figure S1). At the family level, the most common aquatic associated bacterial taxa were members of *Comamonadaceae,* a family of the Betaproteobacteria accounting for 28%, 30%, and 35% bacterial taxa in control, probiotic, and antibiotic waters, respectively. *Mycoplasmataceae* were found in all samples, but at relatively higher abundance in antibiotic challenge water compared to probiotic and control waters. Members of *Oxalobacteraceae* were also found in all sampled tanks but at higher abundance in the probiotic and control tanks relative to the antibiotic tanks. Other notable freshwater-associated bacterial taxa at the family level were *Flavobacteriaceae*, *Pseudomonadaceae*, *Sporichthyaceae* and *Aeromonadaceae* (Figure 2A).

To quantify treatment effects on the aquatic bacterial communities, alpha and beta diversity indices for water samples were compared for the three treatment groups (antibiotic, probiotic, control). Alpha diversity analysis (Chao1, PD) showed no significant differences among the groups (Chao1: KW 5, $P > 0.05$; PD: KW 3, $P > 0.05$). However, our PCoA plot showed clear separation of antibiotic treatment group from the other groups (Figure 2B). PERMDISP (P value < 0.05) and PERMANOVA (F-value: 8.9; R-squared: 0.22; P value < 0.001) results confirmed that the overall community structures were significantly different among the three groups. Pairwise comparison also showed that the three groups are different from each other, but with the probiotic treatment group compared to antibiotic treatment group showing the highest dissimilarity (probiotic- control F: 2.17, $P<0.001$; probiotic- antibiotic F: 2.86, $P< 0.001$; control-antibiotic F: 2.77, $P < 0.001$). Moreover, the average dissimilarity within treatments was higher for the control tanks (73.2%) compared to our probiotic (65.4%) and antibiotic treatment tanks

(61.8%). Jaccard distance matrix analyses showed similar results with significant differences among the groups (Supplementary Figure S2A, Supplementary Tables S3, S4).

Microbial community associated with gut: Firmicutes were the most common phylum for the control (46%) and probiotic (49%) group fish (Supplementary Figure S3A). On the other hand, members of Desulfobacterota were the most common bacteria in the antibiotic treated fish gut microbiomes (Supplementary Figure S3A). We also compared members of Firmicutes phylum among the treatments at the family level. Within the Firmicutes phylum, *Mycoplasmataceae* was the most common gut associated bacterial taxa across all treatments, in addition to other important taxa (Supplementary Figure S3B). For example, control and probiotic treated fish had *Mycoplasmataceae* (control (65%), probiotic (50%)), *Streptococcaceae* (control (30%), probiotic (28%)), and *Lactobacillaceae* (control (2%), probiotic (17%)) present. However, in the antibiotic group, different families were present within Firmicutes phylum (*Mycoplasmataceae* (68%), *Streptococcaceae* (14%), and *Leuconostocaceae* (5%) (Supplementary Figure S3B). At the family level, the most common gut associated bacterial taxa across all treatment groups were members of *Desulfovibrionaceae* (related to Desulfobacterota phylum) and *Mycoplasmataceae* (Figure 3A). While *Streptococcaceae* had high relative abundances in control group, samples in probiotic groups had high relative abundances *Lactobacillaceae.* Moreover, members of *Pseudomonadaceae* had high relative abundances in antibiotic group (Figure 3A). Unlike in the tank water microbiome, *Mycoplasmataceae* was higher in the control and probiotic groups compared to the antibiotic group. At the genus level, we also found two potential fish associated pathogen groups, *Enterovibrio* and *Photobacterium* (from *Vibrionaceae* family), in the fish gut microbiome; however, they were at low abundance based on their read count.

To identify the treatment and parental (dams and sires) effects on the gut bacterial community, alpha diversity indices for gut samples were compared. Alpha diversity analysis (Chao1, PD) for the gut microbiome showed no significant differences among the treatments (Chao1: KW 2.8, $P > 0.05$; PD: KW 3.2, $P > 0.05$), sires (Chao1: KW 6.9, $P > 0.05$; PD: KW 6.8, $P > 0.05$), and dams (Chao1: KW 5.3, $P > 0.05$; PD: KW 8.9, $P > 0.05$). Beta diversity variation was also explored using Bray-Curtis distance matrices and a PCoA plot. The PCoA plot showed weak separation among the samples based on treatments (Figure 3B). PERMDISP and PERMANOVA results confirmed that the overall bacterial community structures were significantly different among the treatments (Table 1). Treatment alone had the highest influence on the gut microbial community (PERMDISP: P value < 0.005; PERMANOVA: Pseudo-F:6.1, P value < 0.05).

Pairwise comparisons also showed that the three treatment groups exhibit significant difference in beta-diversity, with the probiotic versus control treatment samples showing the highest dissimilarity (probiotic- control F: 3.01, P<0.001; probiotic- antibiotic F: 2.85, P< 0.001; control-antibiotic F: 1.52, P < 0.05). Moreover, the average within treatment group bacterial community dissimilarity was higher for the control (82.2%) than the probiotic (77%) and antibiotic treatments (80.5%), indicating that the control group had higher diversity than the other two groups in the fish hindgut. Dams alone did not have significant effects. However, sires had marginal significant effect effects on bacterial community structures (Table 1). Jaccard distance matrix analyses also showed significant differences among the treatment groups for the fish gut microbiome (Supplementary Figure S2B, Supplementary Tables S3, S4).

Association between gut and aquatic microbial community: We evaluated the relationship between the tank water microbiome and the fish gut microbiome. Chao1 and PD (diversity measures) showed significant differences in the species richness of the two sample types; overall, diversity was significantly higher in the water samples than gut samples (P <0.001, Mann-Whitney U test: 2191.5). The PCoA plot (Figure 4) showed clear separation between the gut and water samples. Moreover, PERMDISP and PERMANOVA test also revealed that the clusters showed in PCoA plot were significantly different (PERMDISP: P value < 0.01; PERMANOVA: Pseudo-F: 39.6, P value < 0.05). Additionally, Jaccard distance matrix analyses revealed similar results with significant differences among the treatment groups for fish gut microbiome composition (Supplementary Figure S2B, Supplementary Tables S3, S4).

Treatment effects on the host gut transcriptome.

To determine if antibiotic and probiotic-induced changes in the microbiome led changes in the host gut transcriptome, RNA-Seq was used to determine host transcript levels in the hindgut. Pairwise treatment comparisons resulted in 96 (control vs antibiotic; 35 control upregulated and 61 control downregulated), 105 (control vs probiotic; 61 control upregulated, and 44 control downregulated), 120 (antibiotic vs probiotic; 84 antibiotic upregulated, and 36 antibiotic downregulated) transcripts that were differentially expressed among treatments (Benjamini-Hochberg false-discovery rate (BH FDR) 0.1, $|\log_2 FC| > 0.25$). However, for selecting candidate genes for the OpenArray high-throughput qPCR analyses, we took a conservative approach and we only selected genes with transcripts that were significantly expressed at $|\log_2 FC| > 1$ and FDR P value < 0.05 (Supplementary Figure S4). This decreased the differentially expressed transcripts to 29 (control vs antibiotic), 29 (control vs probiotic), and 27 transcripts (antibiotic vs

probiotic) (Table 2). For the control versus antibiotic group comparisons, the selected genes related to cellular process (e.g., cell activation, cell communication, cell cycle, and cell death) were upregulated and genes related to metabolism and response to stimuli and stress were downregulated in antibiotic group (Table 2). While in the control versus probiotic group comparisons, genes related to regulation of a variety of functions (regulation of meiosis, intracellular protein transport, angiogenesis, transmembrane transporter, cell adhesion, negative regulation of apoptotic process) were downregulated and genes related to post-translation modifications were up-regulated in the probiotic treated fish (Table 2). Moreover, when we compared antibiotic against probiotic group transcription, genes related to cellular process (mostly apoptotic process) were up-regulated in antibiotic group while genes related to cell adhesion, regulation of transcription were up-regulated in probiotic group (Table 2).

OpenArray high-throughput qRT-PCR

The LMM analysis showed PCs 4, 5, 6, 7 and 9 were significantly affected by treatment (Table 3). We identified only those genes whose contributions to the significantly affected principal component axes were important (Supplementary Figure S5) and selected them for analyses. In our analysis we also included tank, body weight, and OpenArray chip ID as random effects to correct for possible technical, environmental, and body size effects. Chip and body weight were not significant for any of the genes and were dropped from our analyses. Sire effects (nested within dam) were not significant after FDR correction. Moreover, a significant tank effect was observed for only one gene (*anxa1*, $p < 0.05$) before FDR correction. We found no significant effects for dam-by-treatment or sire-by-treatment interactions. After including FDR correction into our model, *aifm3*, *manf*, and *prmt3* still showed a significant treatment effect (Table 4).

Correlation between gut bacterial community and host transcriptional profile

Spearman's rank correlation analysis was carried out to evaluate the potential link between bacterial taxon abundance (at the family level) for taxa common to the gut and differentially transcribed genes, while controlling for treatment and family effect. The abundance of *Lactobacillaceae, Bifidobacteriaceae,* and *Aeromonadaceae* were negatively and positively correlated with several gene transcription levels (Figure 5). However, after incorporating Holm-Bonferroni *P* value correction, only *Lactobacillaceae,* and *Bifidobacteriaceae* was negatively correlated with *manf*, and *prmt3* genes (Figure 5, and Supplementary Table S5).

Discussion

Interactions between fish hosts and their microbiomes have been an under-studied area of research, perhaps due to the complexity of the host-microbiome relationship making the detection of specific microbial features that impact the host phenotype challenging. We approached this problem by manipulating gut microbiomes and measured the impact on key candidate gene regulation – such effects are likely mechanisms for microbes to affect host phenotype and health. We found that our treatments resulted in changes in host gene expression patterns, and those changes were mostly related to immune function and cell motility/integrity. By correcting for the direct effects of the treatment, as well as the quantitative genetic effects of family, we showed that changes in microbial communities do lead to changes in host physiology. Given the putative function of the responding genes, our work indicates a likely effect on host fitness as well. Indeed, many recent studies have shown that microbial symbionts are critical biological components for host traits closely associated with fitness, such as immune system development and function (Fuess et al., 2021; Langlois et al., 2021; Rosshart et al., 2017).

This is the first study to consider and compare the impact of probiotics and antibiotics administered to captive fish on the rearing water microbial communities and we found that the aquatic microbial communities in the rearing tanks were significantly influenced by the feed treatments. This was not expected as the fish food treatment itself represented a small proportion of the tank volume, especially given the low flow through water effect. One possible factor is that up 90% of administered antibiotics are excreted in the urine and faeces of the fish, still in the active form (Polianciuc et al., 2020). The common bacterial phyla we report in the tank water were also reported in other studies that showed Proteobacteria, Bacteroidota, Firmicutes are the dominant taxa in water where fish are held (Chiarello et al., 2015; He et al., 2018; Stevick et al., 2019; Uren Webster et al., 2018; Zhang et al., 2019). Nevertheless, we observed significant treatment effects on the rearing water bacterial communities, one possible explanation would be antibiotic-associated diarrhea leading to more fish gut-associated microbial excretion. Another reason could be antibiotic-susceptible taxa being replaced by taxa resistant to antimicrobial agents (e.g., *Mycoplasmataceae* (Firmicutes) (antibiotic (15%), control (1%), probiotic (3%)). Since the aquatic microbiome itself plays a role in maintaining fish health (Blancheton et al., 2013) quantifying the unexpected effects of feed-based treatment on the rearing water is unexpected and important as it may contribute to dysbiosis and poor health outcomes in the fish. Although the negative effects of antibiotics on healthy fish have been reported before, few

studies have considered the effect of antibiotic treatment on the rearing water microbiome. Furthermore, our study showed that probiotic feed treatment also affected the water microbiome. Previous studies showed that treating water with probiotics can improve water quality (Elsabagh et al., 2018; Tabassum et al., 2021).

The microbial communities present in fish rearing water are thought to affect the initial colonization of the fish microbiota during development (Llewellyn et al., 2014; Talwar et al., 2018). However, similar to other studies (Uren Webster et al., 2018; Wu et al., 2018), our fish gut microbiomes were distinct from the water sample microbiomes. This indicates that the fish host gut microbiome is likely largely independent of the water microbial community and that other factors such as diet and host genome may be contributing disproportionally (Talwar et al., 2018).

Our principal goal was to use probiotic and antibiotic treatments to alter the Chinook salmon gut microbiome to determine the potential role of gut microbiota composition variation in host-microbiome interactions. However, we also assessed how the gut microbial community reacted to the treatments. We found that, while fish gut bacterial community alpha diversity was not affected by the treatments, beta diversity was significantly different among all three treatments. Similar results were reported in other studies, indicating community richness (alpha diversity) did not respond to treatment with probiotics and antibiotics, but beta diversity did (Hernandez-Perez et al., 2022; Kokou et al., 2020; Laursen et al., 2017, Rasmussen et al., 2022). One possible reason for this is that using antibiotics does not necessarily mean a reduced diversity of bacterial taxa. Indeed, a review showed that individuals with dysbiosis (potentially caused by treatment) can have even more diverse microbial community than healthy individuals (Berg et al., 2020). For example, Rosado et al (2019) showed that treatment of farmed seabass (*Dicentrarchus labrax*) with OTC caused a decrease in core bacterial community diversity in the gill and an increase in the skin. One reason that our probiotic treatment did not change bacterial community alpha diversity may be we treated healthy fish. Previous studies in human have shown that probiotics in healthy patients (healthy state) does not greatly impact the resident microbial populations (Eloe-Fadrosh et al., 2015; Lahti et al., 2013). In general, external stimuli that affect the intestinal environment can drive a hierarchical series of microbiome responses; resistance, resilience, redundancy or finally dysbiosis−depending on if the disturbance overcomes the intestinal microbial ecosystem (Lozupone et al., 2012; Moya and Ferrer, 2016; Sommer et al., 2017). It appears that the microbial responses to probiotics in our study is either

resistance or resilience, as previous studies have shown that the bacterial communities tended to be more resilient to external stimuli. On the other hand, treatment with antibiotics tends to result in either of resilience, redundancy or dysbiosis. Moreover, apart from the treatment effect, overall, the fish gut microbiome in our study was divided into two clusters and none of our measured factors could explain the two clusters (Figure 3A). This could be due to other unmeasured factors (e.g., sex) that may be contributing to variation in the fish microbiome.

We predicted that the gut microbial community would respond to the treatments through an increase in beneficial gut bacteria (probiotic treatment) or through a decrease in the beneficial microbes with a related increase in the number of potential pathogens (antibiotic treatment). This was based on the expectation that antibiotics can cause dysbiosis in the gut, resulting in elevated levels of opportunistic pathogens (Dethlefsen and Relman, 2011; Francino, 2015), while prebiotics and probiotics are expected to increase the frequency of gut barrier-protecting bacteria such as *Lactobacillaceae* and *Bifidobacteriaceae* (Xiao et al., 2014). In this study, bacteria with potential probiotic properties (*Lactobacillaceae*, *Bifidobacteriaceae*, *Streptococcaceae*) were higher in the probiotic group compared to other treatment groups, as expected. On the other hand, *Pseudomonadaceae* and *Aeromonadaceae* had higher relative abundances in the antibiotic treated fish. Similar patterns of response to probiotics and antibiotics in bacterial community structure and composition have been reported by others (Falcinelli et al., 2016; Kokou et al., 2020; Navarrete et al., 2008; Rutten et al., 2015). For example, Kokou *et al* (2020) showed that after seven days of antibiotic treatment, the European seabass (*Dicentrarchus labrax*) microbiome increased in *Staphylococcus*, *Pseudomonas* genera (Proteobacteria). OTC treatment was reported to reduce gut microbial diversity in Atlantic salmon, while enhancing possible opportunistic pathogens belonging to *Aeromonas* spp. likely due to eliminating competing microorganisms (Navarrete et al., 2008). Moreover, Falcinelli *et al* (2016) showed that Firmicutes, specifically *Lactobacillus* genus, were significantly higher in probiotic treated Zebrafish (*Danio rerio*) larvae relative to controls.

Studies in humans (Qin et al., 2010) and fishes (Boutin et al., 2014) have reported that the gut microbiome varies substantially at the individual and population level, and the transcriptome of the fish gut appears to correlate with this variation (Franzosa et al., 2014; Qin et al., 2010). Moreover, Thaiss *et al* (2016) showed that treatment with antibiotics will change the mouse gut microbiome, and that the microbiome in turn regulates fluctuations in the host transcriptome and epigenome. In our study, we showed that our treatment altered the gut microbiota, then we tested

if these changes were associated with changes in host gene expression. Specifically, we showed that several genes related to cellular processes such as cell activation, cell communication, and cell death were upregulated after treatment with antibiotics in the feed. Although previous studies have shown a direct effect of antibiotic treatment on gene transcription in humans (Ryu et al., 2017), antibiotic treatment had a limited effect on gene expression in germ-free mice (Morgun et al., 2015, Ruiz et al*.,* 2017), providing evidence that the microbiome mediates the effects of orally administered antibiotics on the host. In this study we found that our antibiotic treatment resulted in the upregulation of genes related to cell death. Moreover, bacteria from the Firmicutes and Bacteroidetes phyla were reduced while members of the Proteobacteria phylum increased. Zarrinpar *et al* (2018) showed a similar shift the bacterial community in the mouse cecal; however, a cecal transcriptome analysis showed that the changes in the bacterial community resulted in changes in the expression of genes related to cellular growth and proliferation, as well as cell death and survival pathways. This suggests that colonic remodeling after treatment with antibiotics is directly driving changes in the host transcriptome. Additionally, in our antibiotic treatment group, we showed increased transcription of the *mrp7* (multidrug resistance-associated protein 7-like) gene. Moreover, our qPCR analyses showed upregulation of *aifm3* gene in antibiotic group. A study by Stoddard *et al* (2019) in zebrafish showed that after introducing antibiotics to fish, inflammatory gene transcription was downregulated and apoptotic genes such as *aifm3* were upregulated within 24 hours. Antibiotics are designed to pass the gut barrier and become systemic; however, probiotics are live microorganisms that are not able to pass the lumen barrier. Probiotics can directly modulate host physiology by interacting with host cells (mostly immune cells), and through indirect changes in microbiome composition (Langlois et al., 2021). We showed that genes related to post-translation modifications were over-expressed in the probiotic treatment group, relative to the control and antibiotic treatment groups. Previous studies showed that probiotic diet supplements elicit a proinflammatory response in fish (Nayak, 2010) and honeybees (Daisley et al., 2020) which promotes more effective pathogen clearance and improved disease resistance. In this study we found that our treatment with probiotics indeed changes the bacterial community composition with increased numbers of potential probiotics taxa (*Lactobacillaceae* and *Bifidobacteriaceae*). Moreover, our treatment with probiotics showed fewer genes related to apoptosis process responding, relative to the antibiotics group. However, this was not the case for the control treatment, which was expected as the fish in control group were healthy. Finally,

we noticed that our probiotic treatment did change the expression of several genes related to immune function as reported in other studies (Petrof et al., 2004; Tomosada et al., 2013). For example, Tomosada *et al* (2013), showed that Bifidobacteria strains can have immunoregulatory effect in the intestinal epithelial cells by modulation the ubiquitin-editing enzyme. Moreover, similar to this study, Willms et al. (2022) also showed that beneficial bacteria can promote intestinal angiogenesis in Zebrafish. The precise mechanism of action of probiotics remains to be elucidated, especially in healthy states.

One approach to characterize the bidirectional interactions between the host and the microbiome is to perturb the gut and measure the response of the host (such as in AIMD studies). In this study, we used antibiotics and probiotics to modify the microbial communities within the gut and measured host gene transcription responses to those modifications. We explored this effect using correlation between multiple common bacterial taxa and host gene transcription. The results of that analysis were consistent with a microbiome-mediated effect on the host. We found that specific microbial taxa are affecting the regulation of several host genes, for example, the abundance of *Lactobacillaceae* and *Bifidobacteriaceae* were negatively associated with the transcription of the *prmt3* and *manf* host genes. Previous work has shown that *prmt3* gene as a post-translational modification is involved in a number of cellular processes, such as protein trafficking, signal transduction, and transcriptional regulation (Bedford and Richard, 2005; Choi et al., 2008). Moreover, upregulation of *manf* gene can active innate immune cells and repairing damaged tissue (Neves et al., 2016; Sereno et al., 2017). However, further studies will be required to determine the specific association of *Lactobacillaceae* with *manf* host gene.

The direction of interaction between fish gut and microbiome is not clear, yet it is the basis of the co-evolution of the host with it's associated microbiomes. In this study we experimentally modified the fish gut microbiome and evaluated host gut tissue responses to those perturbation using transcriptome analysis and transcriptional profiling coupled with a controlled breeding design to control for host genome variation. Short term (10 days) perturbation of the juvenile Chinook salmon gut microbiome with antibiotics and probiotics affected the microbiome composition and host gene expression patterns. This study achieved a number of important goals: (1) characterized the effects of antibiotics and probiotics on the aquatic bacterial community (2) characterized juvenile Chinook salmon gut microbiome response to antibiotic and probiotic treatment (3) characterized the host gut tissue transcriptional response to antibiotic and probiotic treatments. We showed that our treatments with antibiotics and probiotics not only changed the

Chinook salmon microbiome (composition), but we also observed significant changes at the gene expression level in the gut tissue of the fish. This study provides insight into a long-standing co-evolved symbiotic relationship between fish gut tissue and its associated microbiome. Moreover, understanding factors influencing the fish gut microbiome and its influence on host health and fitness will help in better sustainable growth for the aquaculture.

Data Accessibility Statement

The raw 16S rRNA gene sequencing data are available at the Sequence Read Archive (SRA) of NCBI with PRJNA872508 BioProject accession number. The RNAseq data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE211372 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211372). For a complete list of packages and code for microbiome analyses, see https://github.com/javad30/Regulation-of-Host-Gene-Expression-by-Gastrointestinal-Tract-Microbiota-in-Chinook-Salmon-Oncorhync.

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Conflict of Interest

The authors declare no conflicts of interest.

Authors' contributions

J.S, S.R.C, D.D.H. conceived and planned the experiments. J.S carried out field work. J.S. carried out the wet laboratory sample preparations and experiments. All authors contributed to

selecting the models and the computational framework for the data analysis. J.S., S.R.C and D.D.H. contributed to the interpretation of the results. J.S., took the lead in performing the research, analyzing data and writing the manuscript. S.R.C, and D.D.H., provided critical feedback and helped shape the research, analysis, and manuscript.

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Table 1. Multivariate statistical testing (PERMANOVA) of effects of treatment, dams, and sires (nested within dams) on microbial community beta diversity (Bray-Curtis dissimilarity matrix).

Source	df	SS	MS	Pseudo-F	P(perm)
Treatment	2	41246	20623	6.1	**0.001**
Dams	5	23505	4700	1.1	0.22
Sires (Dams)	6	24259	4043	1.3	0.06
Res	151	4.6	3107.6	-	-
Total	186	6.5	-	-	-

Table 2. Comparison of gene expression levels and differentially expressed gene distributions between the treatment groups (|log2 Fold Change| > 1 and FDR *P* value < 0.05).

Genes	Gene name	Gene abbreviation	Base Mean	log2 FD	*P* value	padj
Control Vs Antibiotic						
LOC112234113	transmembrane protein 220-like	*tmem220*	40.0	4.68	1.09E-08	0.000
ubr4	ubiquitin protein ligase E3 component n-recognin 4	*ubr4*	149.1	-2.75	5.55E-07	0.005
LOC112248188	annexin A1-like	*anxa1*	123.0	-5.56	6.34E-07	0.005
LOC112241821	kinase D-interacting substrate of 220 kDa B-like	*kidins220*	558.8	-1.12	1.35E-06	0.007
LOC112241820	NTPase KAP family P-loop domain-containing protein 1-like	*nkpd1*	422.9	-1.36	1.20E-06	0.007
LOC112255867	neuronal acetylcholine receptor subunit alpha-3-like	*chrna3*	88.5	1.10	1.73E-06	0.008
LOC112220969	macrophage-stimulating protein receptor-like	*mst1r*	47.2	-3.38	2.10E-06	0.008
LOC112248679	golgin subfamily A member 7-like	*golga7*	6.7	-6.86	1.97E-06	0.008
LOC112245911	*vacuolar protein sorting-associated protein 33A*	*vps33a*	57.2	5.09	3.59E-06	0.009
LOC112216100	*cyclin-dependent kinase 11B*	*cdk11b*	32.7	-4.1	3.38E-05	0.03
LOC112231356	*free fatty acid receptor 2-like*	*ffar2*	21.3	-3.66	4.05E-06	0.009
LOC112259970	vacuolar protein-sorting-associated protein 25-like	*vps25*	12.4	-3.52	3.82E-06	0.009
LOC112258258	septin-8-A	*SEPTIN8*	42.1	3.84	4.24E-06	0.008
xrra1	X-ray radiation resistance associated 1	*xrra1*	2201.2	1.07	6.86E-06	0.011
LOC112246816	HCLS1 binding protein 3	*hs1bp3*	160.1	-4.37	6.66E-06	0.011
nsrp1	nuclear speckle splicing regulatory protein 1	*nsrp1*	21.1	-3.91	6.33E-06	0.011
LOC112239977 *	apoptosis inducing factor mitochondria associated 3	*aifm3*	14.9	-5.00	6.54E-06	0.011
LOC112232613	peroxisomal biogenesis factor 14	*pex14*	17.2	-4.31	7.21E-06	0.011
cfap58	cilia and flagella associated protein 58	*cfap58*	302.4	1.73	1.04E-05	0.014
LOC112218626	WAS protein family homolog 1	*wash1*	58.3	1.22	1.11E-05	0.014
LOC112215983	natriuretic peptide B	*nppb*	34.2	1.61	1.06E-05	0.014
LOC112261371	echinoidin	echinoidin	58.3	1.2	1.18E-05	0.014
LOC112245791	multidrug resistance-associated protein 7-like	*mrp7*	28.4	-4.01	9.57E-06	0.014
LOC112234485	uncharacterized	uncharacterized	192.0	-4.76	1.23E-05	0.015
LOC112243336	SET domain containing 2, histone lysine methyltransferase	*setd2*	86.5	-1.29	1.37E-05	0.016
LOC112251319	phosphatase and actin regulator 1	*phactr1*	23.1	-3.97	2.82E-05	0.030
LOC112265673	serine protease 16	*prss16*	41.2	3.69	4.34E-05	0.043
LOC112249106	piezo-type mechanosensitive ion channel component 1	*piezo1*	41.2	3.69	4.34E-05	0.043
LOC112232636	ER degradation enhancer, mannosidase alpha-like 2	*edem2*	29.8	4.52	4.83E-05	0.047
Control Vs Probiotics						
LOC112242158	Sorting nexin-10A	*snx10b*	7818.8	1.24	9.94E-10	0.000
LOC112232343	Heat shock factor-binding protein 1-like	*hsbp1*	131.3	-6.57	1.91E-08	0.000
LOC112245911	Vacuolar protein sorting-associated protein 33A	*vps33a*	57.2	5.40	3.37E-07	0.002
LOC112246837	COMM domain containing 10	*commd10*	31.4	-5.88	3.12E-07	0.002
LOC112241820	NTPase KAP family P-loop domain-containing protein 1-like	*nkpd1*	422.9	-1.35	5.06E-07	0.002
LOC112220855	COP9 signalosome complex subunit 6	*cops6*	138.4	-5.74	5.03E-07	0.002
LOC112218768	WEE2 oocyte meiosis inhibiting kinase	*wee2*	353.9	1.15	9.19E-07	0.003
LOC112253929	homeobox protein PKNOX1	*pknox1*	18.1	-5.02	8.06E-07	0.003
LOC112234113	transmembrane protein 220-like	*tmem220*	40.0	3.60	1.73E-06	0.004
prmt3*	protein arginine methyltransferase 3	*prmt3*	138.8	-5.25	3.34E-06	0.007
LOC112255055	LSM3 homolog, U6 small nuclear RNA and mRNA degradation associated	*lsm3*	114.8	-7.27	0.00001	0.017
LOC112264620	THO complex subunit 4	*alyref*	138.8	-5.25	0.000003	0.007
LOC112248608	B-cell linker protein-like	*blnk*	37.6	-4.01	3.57E-06	0.007
LOC112246700	cytochrome b-c1 complex subunit 6, mitochondrial-like	*uqcrh*	97.9	4.75	9.35E-06	0.015
LOC112219606	transmembrane protein 38B	*tmem38b*	114.8	-7.27	1.11E-05	0.017
LOC112249934	syndecan-1	*sdc1*	71.6	-3.41	1.27E-05	0.017
LOC112214559	lysophospholipid acyltransferase	*lpcat4*	42.1	3.46	1.27E-05	0.017
LOC112217687	R-spondin-3-like	*rspo3*	90.9	1.38	2.33E-05	0.028
LOC112264739	proteasome subunit beta type-4-like	*psmb4*	97.9	-4.25	3.29E-05	0.034
LOC112261503	sodium-dependent multivitamin transporter	*slc5a6*	291.4	1.80	3.44E-05	0.035
LOC112263481	CD151 antigen	*cd151*	18.5	4.07	3.89E-05	0.037
LOC112232610	phosphogluconate dehydrogenase	*pgd*	155.3	-3.97	4.62E-05	0.040
rabep2*	rab GTPase-binding effector protein 2	*rabep2*	46.8	-3.05	4.56E-05	0.040
sidt2	SID1 transmembrane family, member 2	*sidt2*	19.7	4.66	4.79E-05	0.040
LOC112248204	low-density lipoprotein receptor-related protein 2	*lrp2*	12.9	5.16	5.71E-05	0.045
LOC112242147	uncharacterized	uncharacterized	291.3	1.8	3.44E-05	0.034
LOC112254714	ras-related protein Rab-34-like	rab34b	558.7	-1.03	2.59E-06	0.005
LOC112234485	uncharacterized	uncharacterized	191.9	-5.59	7.11E-08	0.0007
LOC112241821	kinase D-interacting substrate of 220 kDa B-like	*kidins220*	558.7	-1.03	2.59E-06	0.005

Antibiotic Vs Probiotic						
LOC112216146	FERM domain containing 4Bb	*frmd4bb*	24.1	3.93	3.97E-08	0.001
LOC112232343	heat shock factor-binding protein 1-like	*hsbp1*	131.3	-6.47	1.22E-07	0.002
LOC112220855	COP9 signalosome complex subunit 6	*cops6*	138.4	-6.27	2.05E-07	0.002
LOC112248188	annexin A1-like	*anxa1*	123.0	5.74	2.91E-07	0.002
LOC112265459	arsenite methyltransferase-like	*as3mt*	57.5	-4.33	2.39E-07	0.002
LOC112232636	ER degradation enhancer, mannosidase alpha-like 2	*edem2*	29.8	-5.67	3.23E-07	0.002
LOC112218768	WEE2 oocyte meiosis inhibiting kinase	*wee2*	353.9	1.19	1.16E-06	0.004
LOC112232613	peroxisomal biogenesis factor 14	*pex14*	17.2	4.82	1.14E-06	0.004
LOC112252883	transient receptor potential cation channel subfamily V member 5	*trpv5*	160.4	-1.01	1.43E-06	0.005
LOC112222087 *	mesencephalic astrocyte-derived neurotrophic factor	*manf*	43.9	-2.96	1.98E-06	0.006
LOC112246837	COMM domain containing 10	*commd10*	31.4	-5.39	4.10E-06	0.010
nsrp1	nuclear speckle splicing regulatory protein 1	*nsrp1*	21.1	4.00	4.35E-06	0.010
LOC112239977	apoptosis inducing factor mitochondria associated 3	*aifm3*	14.9	5.17	4.30E-06	0.010
LOC112249580	transcription factor 12	*tcf12*	40.7	-1.1	5.96754E-06	0.01
LOC112220311	Occludin a	*ocln*	2096.9	1.05	7.94E-06	0.016
LOC112231356	free fatty acid receptor 2-like	*ffar2*	21.3	3.47	1.10E-05	0.020
LOC112264739	proteasome subunit beta type-4-like	*psmb4*	97.9	-4.59	2.06E-05	0.033
LOC112237710	dispanin subfamily A member 2b	*dspa2b*	780.5	1.89	2.69E-05	0.035
LOC112262831	polyubiquitin	*ub*	687.1	2.32	2.65E-05	0.035
LOC112245658	trafficking kinesin-binding protein 1	*trak1*	36.8	2.52	2.38E-05	0.035
LOC112215983	natriuretic peptide B	*nppb*	34.2	-1.54	2.60E-05	0.035
LOC112218750	protein mono-ADP-ribosyltransferase	*parp12*	39.3	3.13	2.94E-05	0.036
LOC112225266	interferon alpha/beta receptor 2	*ifnar2*	158.6	1.07	3.60E-05	0.043
LOC112217407	protein PML	*pml*	442.7	1.36	4.40E-05	0.048
ano7	anoctamin 7	*ano7*	62.4	-3.31	4.93E-05	0.049
LOC112245441	uncharacterized	uncharacterized	20.3	2.37	4.77E-05	0.049
LOC112225425	T cell differentiation protein 2	*mal2*	155.6	1.05	6.50E-05	0.049

* Indicate that these genes were also significant in our OpenArray high-throughput qRT-PCR analysis.

Table 3: LMM model of PC1-9 (Eigenvalue > 1, and % variance explained > 2%) on the qPCR data for the 48 selected genes test for the effect of treatment.

PCA axes	Type III Sum of Squares	df	Mean Square	F	Sig.
PC1	64.65	2	32.32	1.75	0.17
PC2	5.19	2	2.59	0.44	0.64
PC3	7.91	2	3.95	1.42	0.285
PC4	27.45	2	13.72	9.59	0.0002 ***[a]
PC5	31.93	2	15.96	14.44	1.038e-05 ***
PC6	12.64	2	6.32	5.99	0.0097 **
PC7	12.19	2	6.09	4.44	0.01 *
PC8	3.16	2	1.58	1.64	0.23
PC9	10.62	2	5.31	5.93	0.003 **

a. Signif. codes: $0.01 < P \leq 0.05$*, $0.001 < P \leq 0.01$**, $P \leq 0.001$***

Table 4: Results of the LMM analysis for significance levels for treatment, dam, sire (nested in dam), tank (nested in sire nested in dam) effects for each. Body weight, dam, treatment×dam, treatment×sire effects were nonsignificant before FDR correction and were removed from the model. Treatment was considered as fixed effects, with body weight, dam, and sire effects as random effects. The dependent variable was log transformed ΔC_T.

Genes	Probiotics vs Control	Antibiotics VS Control	Treatment	Sire (nested within dam)	Tank (sire(dam))
uqcrh	0.9	0.14	0.11	0.036 *	0.32
sidt2	0.07	0.17	0.08	0.09	0.43
rabep2	0.05*[a]	0.60	0.015*	0.78	0.45
piezo1	0.06	0.25	0.18	1.00	1.00
ffar2	0.71	0.14	0.09	0.83	0.89
trpv5	0.52	0.88	0.62	0.33	0.98
aifm3	0.04*	0.89	**0.002** **[b]	0.009 **	0.99
ub	0.78	0.14	0.05*	0.62	1.00
dspa2b	0.4	0.20	0.06	0.07	1.00
pml	0.58	0.39	0.60	0.01 *	0.99
nkpd1	0.27	0.33	0.47	0.04 *	1.00
tmem38b	0.41	0.02*	0.07	0.13	0.98
pknox1	0.63	0.44	0.43	1.00	1.00
manf	**0.0001****	0.87	**5.6e-06** ***	1.00	0.14
ifitm3	0.44	0.35	0.25	0.17	0.49
ifnar2	0.4	0.80	0.77	0.02 *	0.99
anxa1	0.57	0.31	0.19	0.13	0.02*
prmt3	**0.0027****	0.16	**0.001** ***	0.37	1.00

a. Significant codes: $0.01 < P \leq 0.05$*, $0.001 < P \leq 0.01$**, $P \leq 0.001$***

b. Significant bold *P* value indicates significant after *P* value correction.

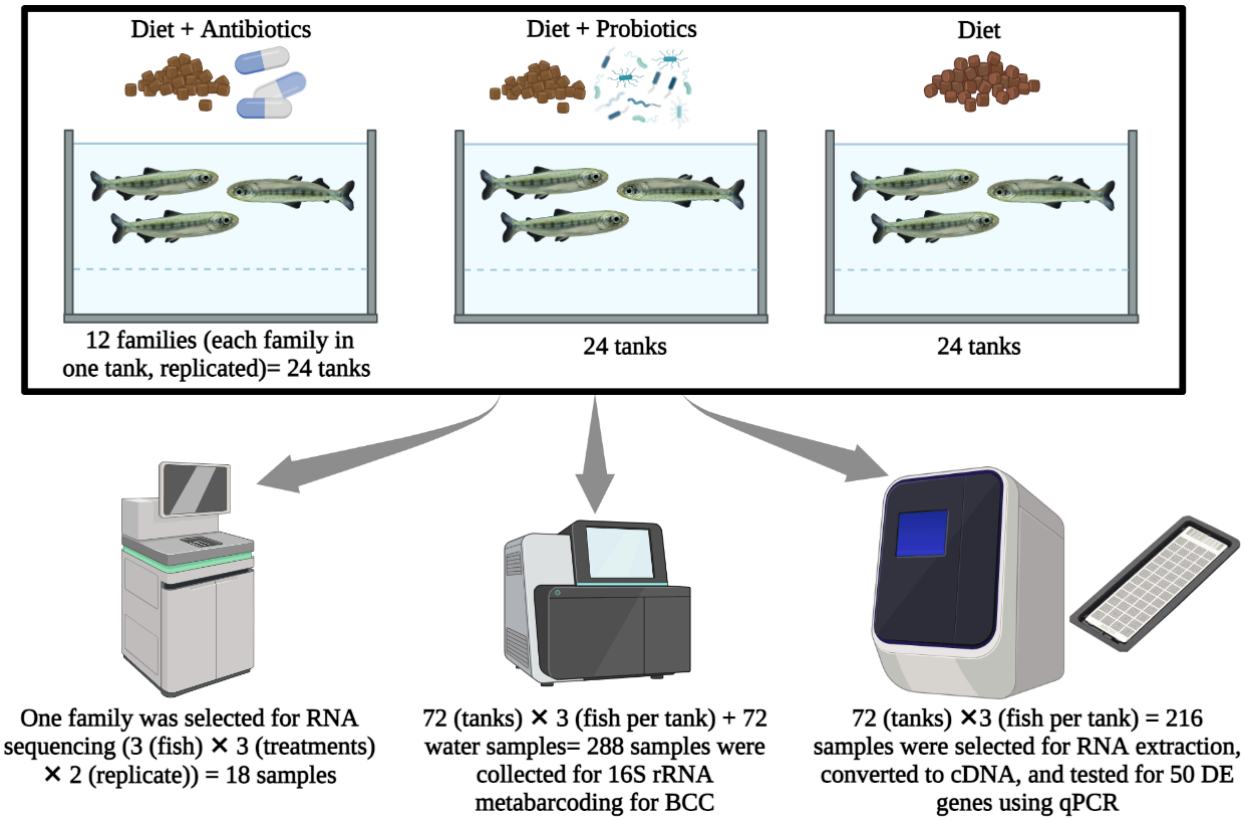

Figure 1: Experimental design and the number of samples collected for each experiment.

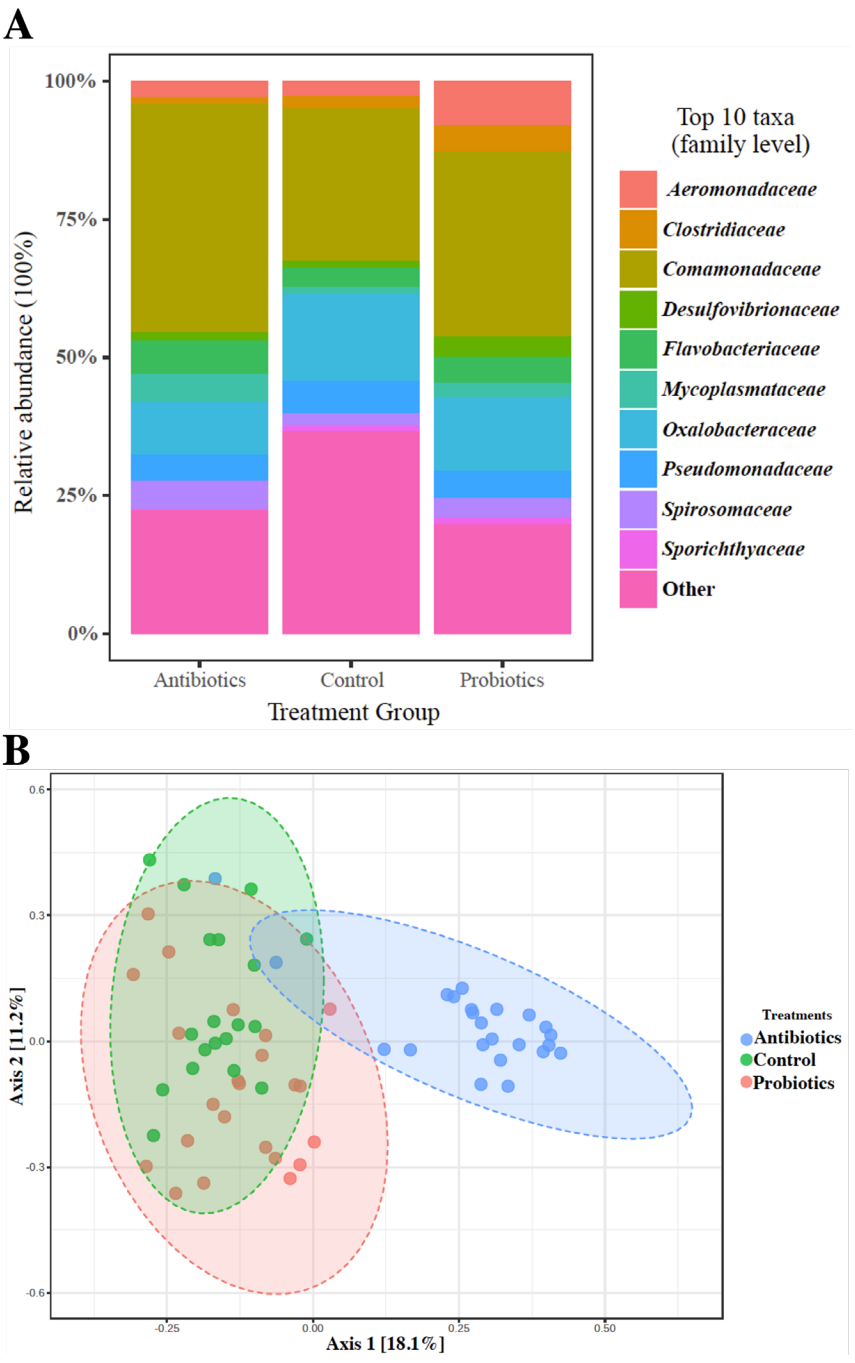

Figure 2: Panel (A) showing relative abundance (top 10) of water bacterial community composition presented at the family level. The 'other' taxa category includes the sum of all bacterial families. Panel B: Scatterplot of the first two axes from the PCoA of the tank water bacterial community where the treated fish were held. Treatment is shown by colour with the 95% ellipses.

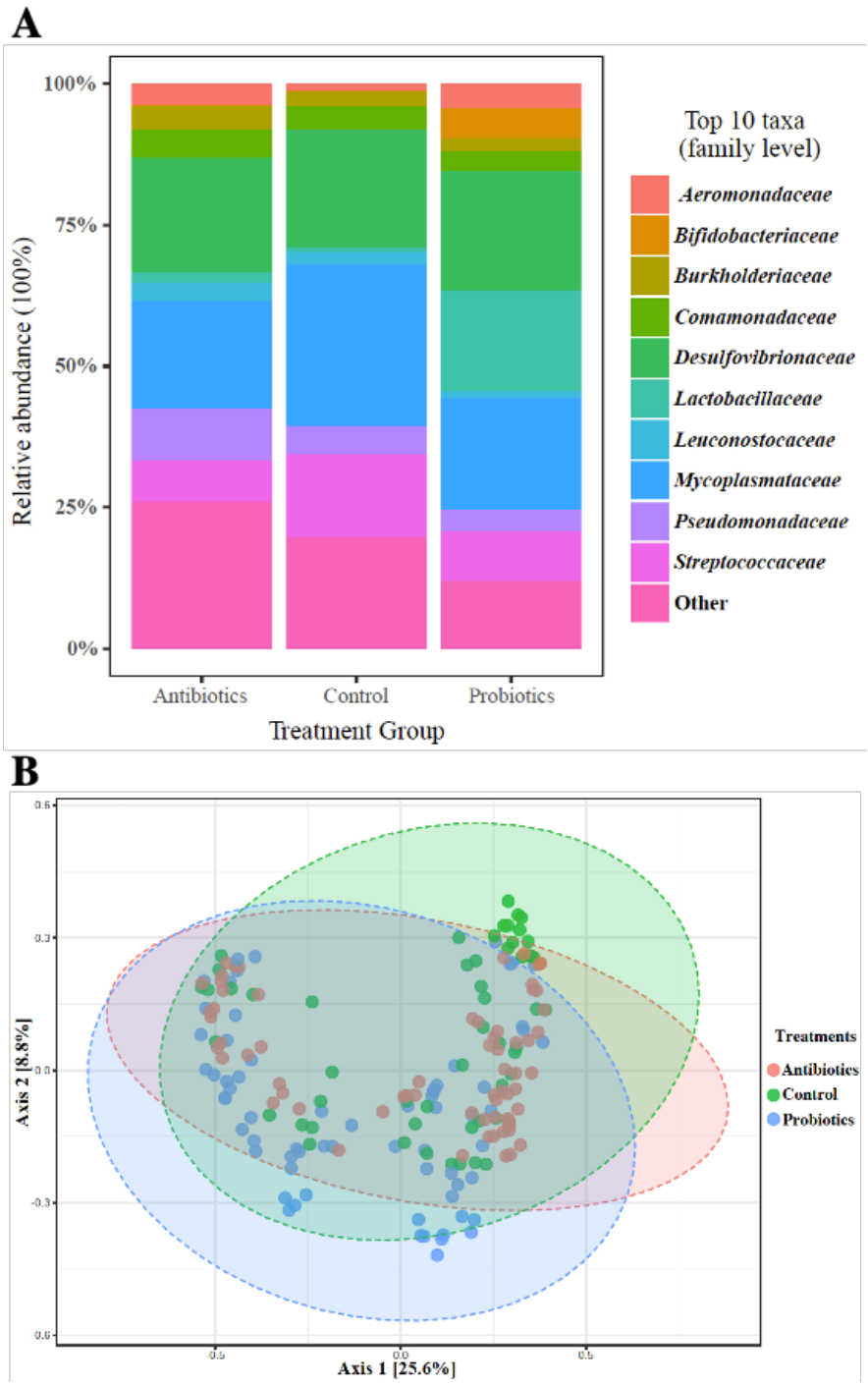

Figure 3. Panel (A) showing relative abundance (top 10) of gut bacterial community composition presented at the family level. The 'other' taxa category includes the sum of all bacterial families. Panel (B) Scatterplot of the first two axes from the PCoA of the Chinook salmon gut bacterial community. Treatment is shown by colour with the 95% ellipses.

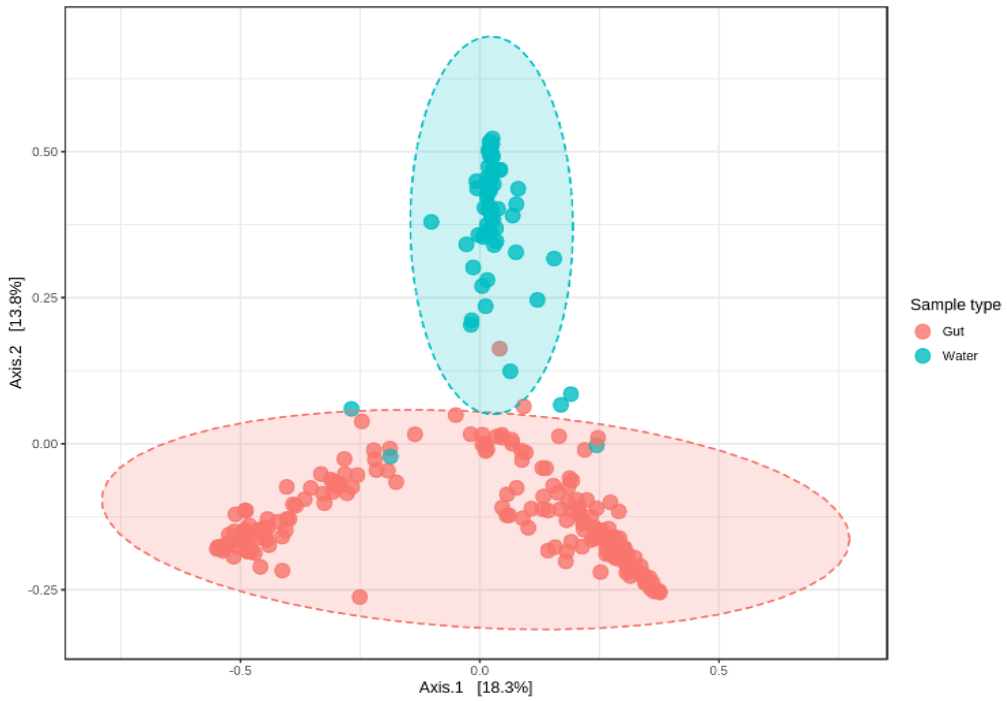

Figure 4. Scatterplot of the first two axes from the PCoA of the Chinook salmon gut as well as water bacterial community. Sample type is shown by colour with the 95% ellipses.

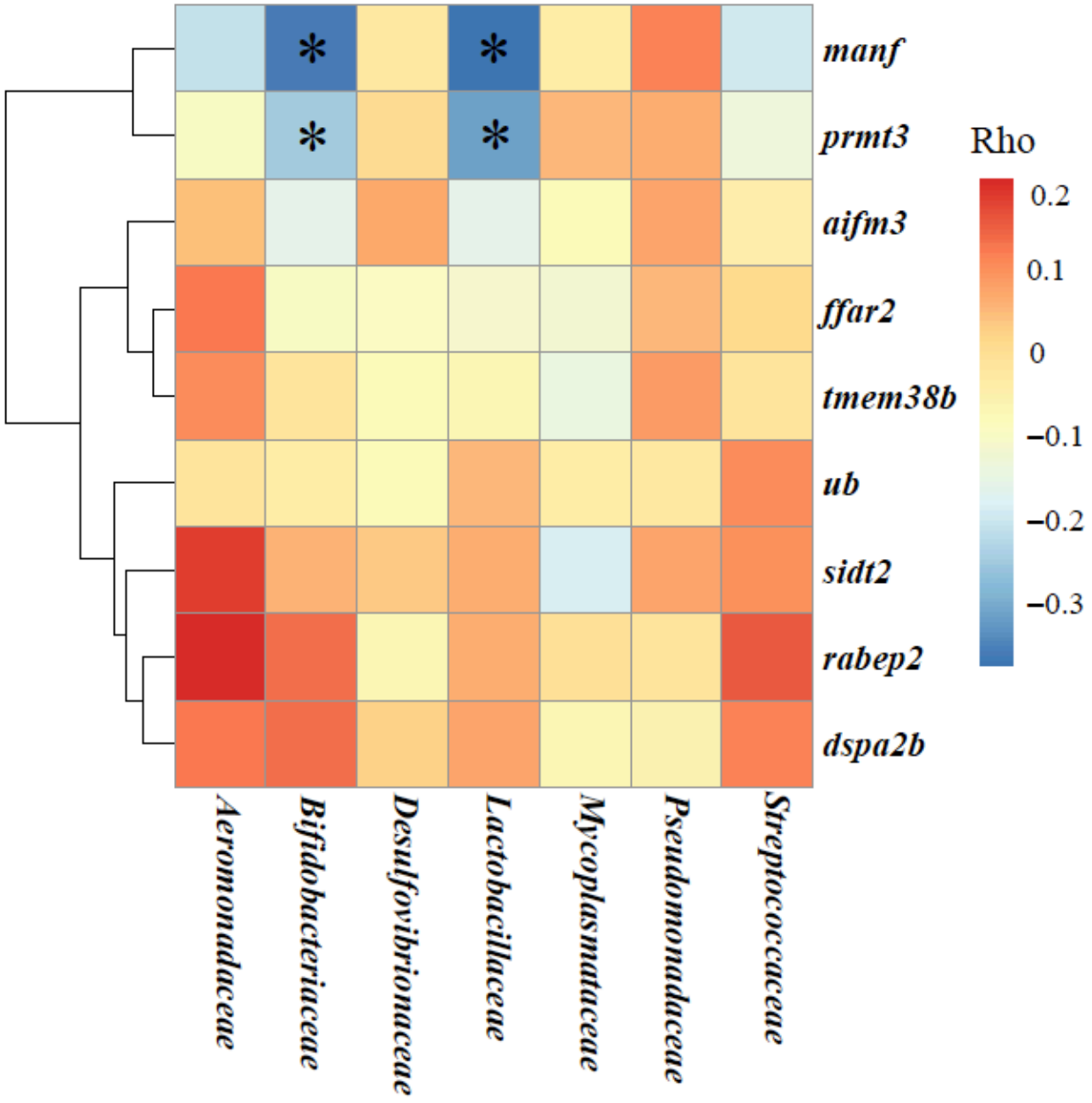

Figure 5. Hierarchical clustering of the 7 core bacterial taxa and association with gene expression. Columns correspond to the 7 core bacterial taxa; rows correspond to 9 selected differentially expressed genes. Red and blue denote positive and negative associations, respectively. The intensity of the colors represents the degree of association between the genus abundance and bacterial taxa are based on Spearman's Rank Correlation coefficient rho. Stars in each square represent significant *P* values (adjusted).