Salmon Gut Microbiota Correlates With Disease Infection Status: Potential for Monitoring Health in Farmed Animals

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Abstract

Background: Infectious diseases cause significant production losses in aquaculture every year. Given the pivotal role played by the gut microbiota in regulating the host immune system, health and physiology, interest has risen in the possibility of controlling the fish health status by modulating the gut microbiota. An altered gut microbiota is often associated with a disease status. However, few studies have examined the association between disease severity and degree of gut dysbiosis, especially when the gut is not the site of the primary infection. Moreover, there is a lack of knowledge on the impact of formalin, a commonly used disinfectant in aquaculture, on the gut microbiome. Here we investigate, through 16S rRNA gene metabarcoding, changes in the distal gut microbiota composition of a captive-reared cohort of Atlantic salmon (*Salmo salar* L.), in consequence of an external bacterial skin infection and the subsequent formalin treatment.

Results: We show that the distal gut of diseased salmon presented a different composition from that of healthy individuals, with an increased relative abundance of strains regarded as opportunistic in sick fish. Conversely, healthy salmon were dominated by a new, yet undescribed, *Mycoplasma* genus. We also observed a positive correlation between fish weight and *Mycoplasma* sp. relative abundance, potentially indicating a beneficial effect for its host. Moreover, we observed that the treatment with formalin, while suited to resolve the external infection, was unable to recover the gut microbiota characteristic of healthy fish prior to treatment, potentially compromising the subsequent health status and growth performances of all treated fish.

Conclusions: We conclude that infectious diseases have the potential of affecting the host gut microbiota at different body sites and that treatment optimization procedures should account for that. The formalin treatment is not an optimal solution from a holistic perspective, we suggest its coupling with a probiotic treatment aimed at re-establishing the original community. Lastly, we have observed a positive correlation of *Mycoplasma* sp. with salmon health and growth performances and, while inviting further research on this microorganism, we also encourage further investigations towards its potential utilization as a biomarker for monitoring health in salmon and potentially other farmed fish species.

Background

The aquaculture market is expanding. As reported by the Food and Agriculture Organization of the United Nations in a 2018 report [1], in 2016 the global aquaculture production was 110.2 million tonnes with a first-sale value of $243.5 Billion (USD), of which 80.0 million tonnes of fish for the food market ($231.6 billion USD), with the salmon industry covering more than 4% of the total fin fish production [2]. In the last half-century, aquaculture has experienced an exceptional growth. In the mid-twentieth-century, capture fisheries were responsible for almost all the fish production. Today only half of the global supply comes from fisheries while the other half comes from aquaculture [1].

The world population is assessed at around 7.7 billion people today and an increase of 2 billion is expected by 2050 according to the 2019 United Nations world population prospect (https://population.un.org/wpp/) [3]. As a consequence, despite the remarkable growth of the aquaculture industries, The United Nations Food and Agriculture Organization forecast a global seafood shortage of 50–80 million tonnes by 2030 [1]. As the world population grows, and the demand for seafood increases, the importance of sustainable food production able to cope with the world demand becomes crucial, and with that comes a growing need to further optimise sustainable farming practices which include better fish health control and enhanced growth performances.

Fish diseases are a cause of major production losses every year in the world of aquaculture [4] and many of them are caused by bacterial pathogenic infections [5]. Infectious diseases have been historically treated with the use of antibiotics. However, with the rise of the antibiotic resistance crisis, sustainable alternatives for disease control are gaining momentum [6].

Given the pivotal role played by the fish gut microbiota in regulating host immune system, health status and physiology [7]–[11], and with an altered gut microbiota often associated with diseases [12]–[14], interest has risen in the possibility of controlling the fish health status and other commercially relevant parameters such as growth performance, by modulating the gut microbiome through the use of pre-, pro- and synbiotics to improve gut health [15]–[25]. However, for an efficient and effective development of new pre- and probiotics a deeper knowledge regarding the healthy gut microbiota of a given species, and its alterations, must be gained. Identifying the factors that govern the gut microbiota and understanding their effect, is the first step for being able to actively establish and maintain a healthy gut microbiota community [11]. Environment [26], diet [27] and host genotype [28] have been demonstrated to play an important role in defining the Atlantic salmon gut microbiota composition. However, up to now, few studies have examined the association between disease severity and degree of dysbiosis in fish, but see [11], [13], [14]. Some diseases common to aquaculture are associated with the infection of multiple strains [29], [30], with the possibility that a bacterial infection could induce the expansions of other opportunistic parasitic strains which may contribute to disease progression. Therefore, understanding disease-induced alterations of the gut microbiota is of crucial importance for understanding the onset and progression of the disease itself and, as a consequence, for the optimization of the treatment. Studies focusing on analyzing the alteration to the gut microbiota during disease progression could give insight into this process and help to develop new strategies for disease monitoring, prevention and control. As an example, the study of the microbiota in the context of a disease could lead to the identification of gut signatures that correlates with the health status of the fish and may serve as useful biomarkers for monitoring gut health and earlier detections of disease.

In water, disinfection treatments are commonly used to treat external parasitosis [4], however, their long term effect on the fish gut microbiota has, to our knowledge, not been investigated. These treatments, while immediately contrasting the expansion of an external pathogen, can potentially cause an alteration of the healthy gut microbiota composition, and compromise the general fish growth performance, by depleting commensal symbionts involved in nutrient metabolism, and overall health status, including

immune system modulation. Thus, understanding how disease progression and common disinfection procedures affect fish gut microbiota is of pivotal importance if we want to improve fish health by actively controlling their microbiota before, during, and after a disease event.

In the present study, a population of captive-reared Atlantic salmon (*Salmo salar* L.) affected by an external parasitosis was treated with a formalin bath treatment (1:4000), one of the most commonly used disinfectants in aquaculture [31], [32]. Notably, individual salmon showed very different susceptibility to the disease. While some manifested extensive skin ulcers along the body (Additional File 1 - Supplementary Fig. 1), others did not show any phenotypic alteration imputable to the disease at the time of sampling.

The present study aims to describe, with 16S rRNA gene metabarcoding, the distal gut microbiota of a captive-reared population of juvenile Atlantic salmon (approx. one-year-old) with individual fish affected to different degrees by an external parasitosis; and the effect of formalin treatment on the gut microbiota. This study provides new insight into both disease and treatment-related alteration of a healthy salmon gut microbiome when reared in captivity in a seawater based flow-through system. Our results should be considered for the development of a novel 16S rRNA gene barcoding based monitoring tool with the potential of detecting pathogenic infections much earlier than e.g. visual symptoms enabling more timely treatments.

Methods

Fish rearing conditions and disease phenotype

The experimental trial was performed at the LetSea land facility (Bjørn, Dønna, Norway) in a seawater based flow-through system heated by heat pump and aerated. Juvenile salmon (*Salmo salar*) were obtained from the commercial hatchery Grytåga Settefisk AS (Vefsn, Norway). Fish were approximately one-year-old and vaccinated with ALPHA JECT micro® 6 (PHARMAQ), a vaccine that protects against furunculosis, vibriosis, cold water vibriosis, winter sore, and infectious pancreatic necrosis. Salmon were initially kept at 12 °C in 24 ppt salinity before being stabilized at 14 °C. The fish were acclimatized whilst being fed commercial feed. Fish were then transferred to 12 replicate different tanks (capacity = 2000L) containing saltwater (33-34ppt of salinity) directly pumped from the sea and subjected to UV treatment for sterilization. Each tank contained between 200 and 300 fish. After the transfer into pure saltwater, a subset of the fish in each tank unexpectedly started to show disease symptoms, specifically the development of large skin ulcers (Additional File 1 - Supplementary Fig. 1). Some fish showed a more severe ulcer phenotype while others seemed in overall good health condition with no external signs of disease. Fish with ulcers were then considered sick, while fish with no visible wounds were scored as “healthy”. The lack of visible wounds is not per se proof of the absence of the causal pathogen but it indicates that these fish were at least at a less progressed stage of the disease and that they might have been able to resist the pathogen for a longer period. As such, the fish scored as healthy still serve as a

useful reference group of more resilient fish for comparison with fish clearly affected by the pathogen or at later stages of the infection.

Diagnostic analysis of disease

To identify the causative agent of the ulcerative disease, a culture based bacteriological analysis from both wound and kidney swabs samples was performed by Vaxxinova Norway AS. Two different kinds of culture media were used: marine agar medium and blood agar with 2% NaCl medium. Sequencing of the V1-V2 hypervariable region of the 16S rRNA gene (primers: 27F AGAGTTTGATCCTGGCTCAG; 519R GWATTACCGCGGCKGCTG [33]) was performed for the identification of the bacterial species.

Water disinfection treatment with formalin

To disinfect the entire water system, formalin was applied (aqueous solution of formaldehyde stabilized with methanol), [31], [32]. For the treatment we used 1L of formaldehyde 38% (38 mg/ml) every 4000L of water (0.5L per tank). Formalin was left in the water to act for 30 minutes before reopening the water flow-through. The treatment included two separate disinfection procedures carried out with a period of four days in between. Each day before treatment the fish were left starving. Samples were collected both before and after the complete formalin treatment.

Distal gut content samples collection

Two samplings were performed at nine days distance in May 2019, one sampling before and one sampling after formalin treatment. A total number of 80 fish were sampled from different tanks. Of these, 40 salmon were sampled before formalin treatment and 40 after treatment. In both cases, 20 healthy and 20 sick fish were randomly picked across replicate tanks (Fig. 1).

Fish were euthanized using an overdose of Finquel MS-222 (Tricaine Methanesulfonate). Within ca 10 min after the euthanization, the abdominal cavity was opened at the ventral midline, and the intestine was aseptically removed. For each salmon, samples from both the distal gut content and the distal gut mucosa were collected (Fig. 1), using scalpels disinfected with bleach 10% and ethanol 70%. Samples were stored in 1.5 ml sterile Lysing matrix E tubes (MP Biomedicals™) containing 1x DNA/RNA Shield™ buffer (Zymo Research). Tubes were kept at room temperature for the transport to the laboratory and then transferred to a -20 °C freezer until DNA extraction.

Length (cm), weight (g) and gutted weight (g) were measured for each fish to investigate possible correlations between the gut microbiota composition and a high growth performance profile of the fish. Information on the presence or absence of visible wounds were recorded and then utilized to assess the health status of each individual fish. The presence of visible wounds was used as a phenotypic marker for the presence of the disease. Fish without visible wounds were considered as in overall good health with no, or little impact, by the pathogen (see above).

Three binary variables can be recognized in our experimental design: 1) healthy vs sick fish, 2) before vs after formalin treatment and 3) type of sample (distal gut content vs distal gut mucosa), for a total of eight (2^3) groups, each one constituted of 20 samples (Fig. 1 and Additional File 2). The above-mentioned groups were used in the subsequent analysis and are hereafter referred to with their acronyms as presented in Fig. 1.

DNA extraction and quality control

DNA extraction and purification from both distal gut content and distal gut mucosa samples was performed with an in-house extraction protocol as described in the Additional File 3. DNA concentration was assessed with Qubit fluorometric 3.0 quantification (Thermo-Fisher Scientific), following the manufacturer's recommendations.

Real-time PCR (qPCR) was performed on all extracts prior to metabarcoding, to optimise the subsequent metabarcoding process. Specifically, all DNA extracts were pre-screened using SYBR Green qPCR [34] with both primer sets to I) screen for contamination in extraction negatives, II) identify the potential presence of PCR inhibitors, and III) optimise the cycles needed for metabarcoding PCRs. qPCRs were performed in 21 µl reactions containing 2 µl DNA template, 9.5 µl of AccuPrime SuperMix II (Invitrogen), 6.5 µl ddH_20, 0.5 µM 16S forward primer, 0.5 µM 16S-reverse primer and 1 µl of SYBR Green/ROX solution (Invitrogen). qPCR amplifications were performed on an Mx3005 qPCR machine (Agilent Technologies) with the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 20 s, and 68 °C for 40 s. Using serial dilutions of the DNA template (1:10 and 1:20) we tested for the presence of contaminants (e.g. excess of host DNA), responsible for PCR inhibition [35]. For some samples only the dilutions amplified. In those cases, the 1:10 dilution was selected for the subsequent PCR (Additional File 4). Negative controls were included in every qPCR reaction.

The Ct values obtained from the qPCR (Additional File 5) were also used to inspect relative differences in the microbial biomass of different samples or groups of samples, with the assumption that the 16S rRNA gene can serve as a proxy for the actual abundance of microorganisms. We accounted for a potential bias introduced by differences in the 16S rRNA gene copy number among OTUs (see below).

Metabarcoding and sequencing

For 16S rRNA gene profiling, the following primers were used: 341 F (5'-CCTAYGGGRBGCASCAG-3') and 806 R (5'-GGACTACNNGGGTATCTAAT-3'), to amplify the V3-V4 region of the 16S rRNA gene [36]. PCR was performed in 0.2 ml PCR tubes using the same reaction composition used for qPCR with the exclusion of the SYBR green and ROX dyes. Tagged primers were used in different combinations to allow multiplexing of samples during sequencing. According to the qPCR results, some distal gut content samples were subjected to 30 PCR cycles of amplification while the others got 35 PCR cycles. For the distal gut mucosa samples, some were subjected to 35 PCR cycles while others were given 40 PCR cycles (Additional File 4). Samples from the gut content amplified better than those from the mucosa (see Additional File 5). PCR was performed in triplicates under the following conditions: denaturation at 95 °C

for 5 min followed by the determined number of cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s and extension at 68 °C for 40 s. After the completion of the cycles, samples were left at 68 °C for 10 minutes for final extension and then cooled to 4 °C. Two PCR blanks were included in all PCR reactions with ultrapure water replacing the DNA template. To reduce the risk of contamination, pre- and post-PCR products were handled in two different laboratories designated for pre-PCR setup and post-PCR processing, respectively and PCR master mix solutions were prepared in a designated DNA template-free laboratory. PCR products were visualized using 2% agarose gel electrophoresis (GE) to check the amplification products quality and amount. All the samples in one PCR replicate have been pooled prior to library preparation. To reduce bias introduced by differential amplification between samples, PCR products were pooled at approximately equimolar ratios determined by gel band strength on the agarose gel. Extraction and PCR blanks were included in the pools for downstream quality filtering, but in a non-equimolar fashion to avoid excessive dilution. PCR replicates were purified through SPRI bead purification [37], with a beads-to-sample ratio of 1X, two washing steps in 0.5 ml of ethanol 80% and elution in 35 µl of EB Elution Buffer (10 mM Tris-HCl). DNA concentration measurement was performed with Qubit 3.0 (Thermo-Fisher Scientific), following the manufacturer's recommendations. We generated following the Tagsteady protocol [38] seven sequencing libraries, including three PCR replicates from the distal gut content samples, three PCR replicates from the distal gut mucosa samples and one library blank made of ultrapure water. Tagsteady is a PCR-free Illumina library preparation protocol specifically developed for metabarcoding studies to avoid false assignment of sequences to samples [34]. Indexed library quantification was performed using NEBNext® Library Quant Kit for Illumina® (NEB, New England Biolabs), following the manufacturer's recommendations. 300 bp paired-end sequencing of the amplified V3-V4 region of the 16S rRNA gene was performed at the Danish National High-Throughput Sequencing Center, University of Copenhagen, Denmark, using an Illumina MiSeq platform with reagent kit v3, 600 cycles.

Bioinformatic data processing

Raw reads were quality filtered and de-multiplexed prior to downstream analyses. Read quality was initially checked with FastQC [39] (Additional File 6). Sequences were subjected to trimming with AdapterRemoval [40]. Adapters were removed, together with low-quality bases (minquality = 28). Only sequences with a minimum length of 100 bp were retained. AdapterRemoval was also used to merge overlapping paired-end sequences, obtaining in this way sequences for the entire 16S rRNA gene V3-V4 region covered by the primers. Reads within each amplicon library were demultiplexed and filtered using Begum (https://github.com/shyamsg/Begum) a modified version of DAMe [41]. Singletons were removed and only sequences present in at least two out of three PCR replicates were maintained for downstream analyses. Merged read pairs were further filtered for their length, conserving only sequences with a length between 380 and 480 bp, discarding the unmerged reads.

Remaining sequences were then used to detect OTUs. OTU clustering was performed with SUMACLUST, using 97% similarity threshold [42]. The use of a higher clustering threshold didn’t affect the results and conclusion of the present study, however since it ensures an improved resolution, it’s utilization should

always be taken into consideration [43]. Begum was used to convert the sequences in a suitable format for the clustering and to generate an OTU abundance table from the SUMACLUST output (Additional File 7). After the OTU table was generated, sequences were blasted using QIIME (version 1.9.1) against the NCBI nucleotide (nt) database for taxonomy assignment (Additional File 8).

Contaminants identification and removal

We identified and removed sequences originating from putative contaminants. To identify contaminants, all the DNA extraction and PCR amplification negative controls were included in the sequencing. The compositional profile of the negative controls was used to identify contaminants (Additional File 1 - Supplementary Fig. 2). To avoid removal of genuine OTUs present in the negative controls as a consequence of cross-contamination, all OTUs present in the negative controls were further investigated through BLAST search and, in some cases, phylogenetic analysis. Among all OTUs detected in the negative controls, only OTU2 (an unknown *Mycoplasma* genus) could be clearly assigned to fish gut or fish-related environments and was therefore retained. All other OTUs were treated as contaminants and filtered out.

For the unknown *Mycoplasma* genus a maximum likelihood (ML) phylogenetic tree was built (Additional File 1 - Supplementary Fig. 3). The sequence of the unknown *Mycoplasma* was blasted against NCBI database with blastx. A total of 28 sequences retrieved from GenBank were selected to be included in the tree, including 15 sequences obtained from fish gut or fish-related environments (such as fish farm sediments). *Mycoplasma mobile*, a fish gill pathogen that showed to diverge from all the other sequences, was included as an outgroup. The 29 sequences were aligned with MUSCLE [51] and the multiple alignment was used to build a ML neighbor-joining (NJ) phylogenetic tree with MEGA5 [52]. A model selection test was performed with MEGA5 to test for the optimal substitution model. The General Time Reversible (GTR) substitution model retrieved the lowest BIC scores (Bayesian Information Criterion) and was therefore considered the model that better described the substitution pattern. The ML-NJ-phylogenetic tree was then constructed using the GTR substitution model and 1000 bootstrap tests.

To further support the non-biological origin of the removed OTUs we utilized patterns of co-occurrence between the OTUs (Additional File 1 - Supplementary Fig. 4), their prevalence in low biomass samples (Additional File 1 - Supplementary Fig. 5) and support from previous literature on reagent contamination [44]–[46]. Remarkably, almost all the OTUs found in the negative controls corresponded to well-known reagent contaminants. Spearman correlation coefficient between OTUs (after rarefaction) was calculated in R (version 3.6.3) [47], [48] and plotted as a heatmap with the corrplot R package [49] (Additional File 1 - Supplementary Fig. 4). The qPCR based Ct values for each sample (Additional File 5) were incorporated in the analysis as a relative proxy for sample biomass to support identified contaminants. Since reagent contaminants are known to mostly affect samples with low microbial biomass [44]–[46], [50] we visualized this correlation by plotting the contaminants relative abundances (calculated as the sum of all the putative contaminants relative abundances) against samples Ct values using the “ggscatter” function

(ggplot2 R package) [51]. We included OTUs recognized as genuine, OTU1 (*Aliivibrio sp.*) and OTU2 (*Mycoplasma sp.*), for comparison (Additional File 1 - Supplementary Fig. 5).

Data normalization and diversity analysis

After contaminants removal, also mitochondrial and chloroplast sequences were manually removed. Samples were then normalized by sub-sampling to a depth of 4,000 reads. All samples with less than 4,000 reads were discarded. In this way, 47 samples out of 160 were excluded. The remaining 113 samples were used for the subsequent analysis. Diversity analyses were conducted, using the hilldiv R-package [52] in Rstudio version 1.2.5033 [47], [48].

Microbial composition analysis

Stacked bar plots representing the microbial composition of the different groups or samples were generated using phyloseq [53], vegan [54] and ggplot2 [51] R packages. We used the gplots package “heatmap.2” function [55] and RColorBrewer [56] to create a heatmap representing OTU abundances among all eight groups. A heatmap dendrogram representing beta diversity among groups was calculated using Jaccard distance metric in vegan. We used Wilcoxon rank-sum test to assess differences in relative abundance of OTUs among groups of samples.

Correlation between microbiota and fish growth performances

To investigate potential correlations between the microbiota and fish growth performances we calculated the Spearman’s correlation coefficients between fish weight and the relative abundance of the most abundant OTUs (*Aliivibrio* sp. and *Mycoplasma* sp.). Spearman correlations were calculated and plotted using ggpubr [57] and ggplot2 R packages. Condition factor K (K value), a normalization of fish weight according to its length, was calculated for every fish (with parameter N = 5 as suited for salmonids) [58], and included in the analysis.

Fish were then grouped according to *Aliivibrio* sp. and *Mycoplasma* sp. relative abundances in their microbiota. Salmon with a percentage of *Aliivibrio* sp. higher than 80% (25 fish in total) were pooled together in one group and salmon with a percentage of *Mycoplasma* sp. higher than 80% (37 fish in total) were pooled in another group. All the other samples were discarded. After testing for prerequisites, we used a Welch's t-test to test for differences in the mean fish weight between the two groups.

Comparison of relative microbial biomass

To reveal variations in microbial biomass among groups we relied on qPCR Ct values. Ct values are inversely proportional to the amount of target DNA in the sample, meaning that in microbial metabarcoding studies the Ct value increases as the amount of microbial biomass in the sample decreases. The assumption is that the 16S rRNA gene can serve as a proxy for the actual abundance of microorganisms. A major deviation from this assumption can be introduced by differences in the 16S rRNA gene copy number among microorganisms. We used qPCR Ct values as a proxy for the samples

microbial biomass and tested for differences in the qPCR Ct values among groups with an ANOVA coupled with a Tukey's HSD post-hoc test for pairwise comparisons in R (version 3.6.3) [47]. To ensure that the observed differences were not an experimental artifact, we checked that our conclusions were robust even when accounting for differences in 16S rRNA gene copy number between *Mycoplasma* sp. and *Aliivibrio* sp., the two OTUs dominating our dataset (see below).

We utilized the information on EzBioCloud database (https://www.ezbiocloud.net/) (Accessed 28 July 2020) [59] to estimate the 16S rRNA gene copy number in *Aliivibrio* sp. and *Mycoplasma* sp. Median values for both genera were selected as an approximation of the real copy number value of our OTUs. *Aliivibrio* genus has a median value of 12, while *Mycoplasma* genus of 2. We then calculated a Ct value correction coefficient as log2(12/2) and, for each sample, we multiplied this value for the *Aliivibrio* sp. relative abundance and added the result to the sample Ct value:

Corrected Ct value = Ct value + (log2(a/m) * *Aliivibrio* relative abundance)

a = *Aliivibrio* genus median 16S rRNA gene copy number

m = *Mycoplasma* genus median 16S rRNA gene copy number

This correction does not account for changes in the OTUs relative abundances as a consequence of the different copy number, which, if included, would reduce the bias by reducing the *Aliivibrio* sp. relative abundance. Therefore, the applied correction has to be intended as a conservative approach to test the robustness of the Ct values based observations and demonstrate they are not a 16 rRNA gene copy number artifact.

Data and scripts used for the analysis are available at the GitHub repository: https://github.com/DavideBozzi/Bozzi_et_al_2020_analysis.

Results

Identification of the causative disease agent

The bacteriological analysis led to the identification of *Tenacibaculum dicentrarchi* as the most likely causative agent of the ulcerative disease. *Tenacibaculm* is a known emerging pathogenic genus causing ulcerative disease (Tenacibaculosis) in salmonids [60]–[65]. *Vibrio tapetis*, a known pathogen of cultured clams where it causes brown ring disease [66], was also isolated from the wounds and kidney samples. *V. tapetis* has been previously isolated from diseased fish [67]–[69] but is not regarded as a primary pathogen for fish [70].

Taken together, these results and previous observations [70] suggest that *Tenacibaculum dicentrarchi* is most likely the disease causative pathogen in the investigated salmon cohort, while *Vibrio tapetis* might be an opportunistic strain that has expanded, taking advantage of the *T. dicentrarchi* infection and the weakened host's immune system.

The distal gut microbiota is characterized by low alpha diversity

In total, 13.7 million reads were generated by the Illumina MiSeq sequencing platform for the distal gut content and the distal gut mucosa samples. After quality inspection and trimming reads were clustered into 130 OTUs using a similarity threshold of 97%. After taxonomy assignment, the validity of the observed OTUs was investigated, in order to identify and exclude potential contaminants from the subsequent analysis (see methods). Contaminants reads, as well as chloroplasts and mitochondrial reads were removed and then samples were rarefied to 4000 reads per sample. In this way, 47 of the original 160 samples were discarded. For the remaining 113 samples, the contaminants removal procedure and the rarefaction process reduced the total number of OTUs to 65. The rarefaction process may lead to the loss of low abundant OTUs and reduced richness values and should therefore be avoided in studies interested in observing low-abundant OTUs. In those cases, other methods of data normalization should be taken into consideration [71], [72].

The relative abundance of OTUs was highly uneven, with just two OTUs being highly abundant across all samples: *Aliivibrio* sp. and an unknown *Mycoplasma* genus. These two OTUs alone accounted for 99.68% of the total number of reads after filtering and normalization.

The alpha diversity analysis highlighted a remarkably low microbial biodiversity in the investigated salmon intestine (Additional File 9). Samples were clustered according to the eight groups defined in Fig. 1 and mean alpha diversity values and standard deviations for each group, both as richness and Shannon index, were calculated (Table 1). Given the highly uneven microbial relative abundances, Shannon index values are expected to better describe the system [73]. The Shannon index values highlight the striking pattern characterizing the investigated samples. Many of them are dominated in the composition by just one OTU, either *Aliivibrio* sp. or the new *Mycoplasma* sp., with few samples presenting both OTUs at high relative abundances.

Table 1
Richness and Shannon index mean values and standard deviations for the eight groups (abbreviations defined in Fig. 1).

	CHB	CSB	MHB	MSB	CHA	CSA	MHA	MSA
Richness	2.05 ± 1.17	2.37 ± 1.57	3.55 ± 2.58	2.33 ± 1.72	4.62 ± 3.62	3.71 ± 5.08	1.54 ± 0.52	3.11 ± 2.32
Shannon index	1.1 ± 0.2	1.13 ± 0.24	1.09 ± 0.18	1.05 ± 0.15	1.4 ± 0.49	1.45 ± 0.43	1.02 ± 0.04	1.05 ± 0.07

BLAST search of the *Aliivibrio* sp. sequence did not identify the specific species since the sequence recovered an exact match (100% of similarity) with two, closely related [74], *Aliivibrio* species: *Aliivibrio logei* and *Aliivibrio salmonicida*, the latter being a well-known pathogen causing cold water vibriosis in salmonids [75].

Conversely, the *Mycoplasma* sp. sequence is part of a yet undescribed genus in the Mycoplasmateceae family. Interestingly, our sequence showed to be phylogenetically related to other *Mycoplasma* species identified in fish gut samples [76]–[80] which phylogenetically clustered together (Additional File 1 - Supplementary Fig. 3), pointing to the existence of a genuine fish-associated *Mycoplasma* genus.

The distal gut microbiota of healthy and sick fish differs in both composition and total microbial biomass

Relative abundances of the two most abundant bacteria, *Aliivibrio* sp. and *Mycoplasma* sp., have been investigated for both the single samples (Additional File 1 - Supplementary Fig. 6) and the eight groups (Fig. 2). All other OTUs constituted less than 0.5% of the total number of reads and were clustered into a single category “others”. In accordance with the alpha diversity results, many samples were either dominated by *Aliivibrio* sp. or *Mycoplasma* sp., with few samples being characterized by both OTUs, many of which were samples from the distal gut content of both healthy and sick fish after formalin treatment. Figure 2 depicts the OTUs relative abundance across groups. Beta diversity among groups has been computed with Jaccard distance metric and groups have been plotted according to their beta diversity clustering.

A striking difference in the microbiota composition between healthy and diseased salmon was observed. In healthy fish the relative abundance of *Mycoplasma* sp. was higher in percentage when compared to sick fish and vice versa (Fig. 2). The observed difference in *Mycoplasma* sp. and *Aliivibrio* sp. relative abundances between healthy and sick fish was statistically significant (Wilcoxon rank-sum test: $p < 0.05$). Some individual outlier samples were present, probably due to errors in the initial phenotypic characterization (Additional File 1 - Supplementary Fig. 6). It is possible that fish initially evaluated as healthy were affected by *Tenacibaculum dicentrarchi*, but that they had not developed external ulcers yet.

Beta diversity among groups calculated as Jaccard distance metric, also showed that CHB and MHB cluster together, indicating that the healthy fish gut microbiota presents a distinct compositional profile that differs from groups affected by the disease and/or the formalin treatment. Together, the Shannon index and the relative abundances observations suggest that, in the present study, before formalin treatment, the healthy fish microbiome is dominated by the unknown *Mycoplasma* sp., while the sick fish gut microbiota is dominated by *Aliivibrio* sp. and that this difference is visible from both the gut content and the gut mucosa samples.

We further investigated possible differences in the gut microbial biomass of healthy and diseased salmon. Differences in mean Ct values among groups were tested with ANOVA coupled with a Tukey’s HSD post-hoc test for pairwise comparisons (Fig. 3). Statistically significant differences were observed when comparing CHB with CSB and MHB with MSB. In both cases the healthy samples presented higher Ct values than their diseased counterpart. This indicates that healthy salmon tends to have lower microbial biomasses than sick ones, pointing to a disease-associated increase in the absolute microbial biomass in the gut.

Taken together compositional and abundance data indicates that the distal gut microbiota of healthy salmon was colonized almost entirely by *Mycoplasma* sp. while in fish affected by the external *T. dicentrarchi* infection there was an expansion of *Aliivibrio* sp. relative abundance which also corresponded to an increase in absolute abundance, with the sick fish being characterized by higher *Aliivibrio* sp. relative abundance and higher microbial biomass in the distal gut.

High growth performance is correlated with a *Mycoplasma* sp. dominated gut microbiota

We checked the presence of a possible correlation between specific OTUs and other relevant phenotypic traits: fish weight and condition factor K [58]. To avoid that the effect of formalin treatment might interfere with potential correlations, only samples before formalin treatment were considered. A similar pattern was observed when using fish weight or condition factor K. Fish weight showed to be positively correlated with *Mycoplasma* sp. relative abundance, and negatively correlated with *Aliivibrio* sp. relative abundance (Fig. 4). Results based on the condition factor K showed a similar pattern (Additional File 1 - Supplementary Fig. 7).

Fish with high *Mycoplasma* sp. relative abundance were statistically bigger (mean = 207.6 ± 18.4 g) than fish with high *Aliivibrio* sp. relative abundances (mean = 187.1 ± 22.4 g), (Welch's t-test: $p < 0.001$).

Together with the previous observations, this indicates that the *Mycoplasma* genus is associated with the gut microbiota in salmon exhibiting higher growth performances and a potentially enhanced resistance to pathogenic infections.

The distal gut mucosa harbors lower relative microbial biomass than the content and is more affected by *Aliivibrio* sp. colonization.

Interesting compositional differences were observed between the mucosa and content gut microbiota profiles. Mucosa samples have a higher *Aliivibrio* sp. relative abundance compared to the content samples in the groups affected by the disease and/or the formalin treatment (Fig. 2). This spatial difference between sample types indicates that the *Aliivibrio* sp. efficiently colonizes the mucosal tissue, in line with what is expected from pathogenic microorganisms, whose success in the infection largely depends on their ability to adhere to host cells and colonize the host tissues [81].

We also observed a signal of different biomass levels between microbiota originating from mucosa and content samples. Statistically significant differences (Tukey's HSD post-hoc test: $p < 0.05$) were observed in the comparison between the mean Ct values of distal gut content groups and their distal gut mucosa counterparts, with the mucosa always presenting a higher mean Ct value (Fig. 3). This suggests that the distal gut mucosa is characterized by a relatively lower microbial biomasses than the distal gut content, an observation which is in accordance with the fact that only distal gut mucosa samples were affected by reagent contamination, indicating that in some fish the mucosa tissue might have harbored an incredibly low microbial concentration.

Formalin treatment has no detectable impact on the gut microbiota

Formalin disinfection treatment is a commonly used procedure which effectively controls external bacterial infection. However, its effect on the gut community has never been investigated. This becomes even more important in light of the fact that the primary infection can likely enable the expansion of other opportunistic and potentially pathogenic strains by weakening the fish immune system.

In our study, the gut microbiota composition of the formalin treated salmon, both healthy and sick, seems to mimic the composition of the affected ones before treatment, with an increased relative abundance of *Aliivibrio* sp. (Fig. 2).

To further investigate whether the formalin treatment directly modified the microbial gut composition, we checked for a possible effect of the treatment on the gut microbial biomass. Here we hypothesize that if the disinfectant is affecting the gut microbiome, a reduction on the microbial concentrations might be expected. However, no such reduction was observed in our data. The comparison of the mean Ct values of the groups, before and after formalin treatment showed no significant differences in the microbial abundance.

Discussion

We set out to investigate the gut microbiota composition of captive reared Atlantic salmon affected to different degrees from an external bacterial infection and the effect exerted by a water disinfection treatment with formalin on the gut microbiota itself.

From the bacteriological analysis of the wounds swabs it was possible to identify *Tenacibaculum dicentrarchi* as the most plausible cause of the ulcerative disease (Tenacibaculosis). *Vibrio tapetis* was also identified from wound and kidney swabs. *Vibrio tapetis* is expected to be an opportunistic species, taking advantage of the weakened immune system of the host to expand, as previously observed [70]. The disease developed after the juvenile salmon (approximately one-year-old) were moved from freshwater to saltwater, pointing to a possible role of the change in salinity in triggering the Tenacibaculosis in accordance with previous studies describing changes in fish skin and gut microbiome in response to change in salinity [82]–[85].

The distal gut microbiota of the investigated salmon was characterized by low levels of alpha diversity in particular compared to previous studies on salmon [13], [26], [86]–[88]. Almost all fish were characterized by only one, highly abundant, OTU, with few samples being characterized by two abundant OTUs. The two most enriched OTUs were *Aliivibrio* sp. and a new, yet undescribed, *Mycoplasma* genus, which together constituted 99.68% of the filtered reads. The low values of richness observed in the salmon distal gut in the present study could potentially be explained by the very strict filtering that we applied to the sequence data so as to minimize the chance of overinflation of OTU richness from contamination. Alternatively, it could be a true biological phenomenon, for example explained by the impact of relatively sterile captive rearing conditions in the microbiota ontogeny process. The water sterilization procedures aimed to control pathogen infections may also compromise the colonization of the fish gut by other, potentially beneficial, bacteria. This, combined with a generally more uniform diet, might be responsible

for the lower levels of alpha diversity observed in the investigated salmon when compared to their wild counterparts [87].

However, low levels of biodiversity are usually observed also in wild adult salmon, indicating a natural reduction in the microbiome alpha diversity during life-cycle stage progression, with the lowest values observed in marine adults [89]. This suggests that high levels of alpha diversity are not necessarily beneficial in adult salmon in contrast to what is often observed in mammals [90], and that a relatively low microbial biodiversity should be expected also in healthy salmon.

A comparison between healthy and sick fish showed that the former were characterized by higher *Mycoplasma* sp. relative abundances, while the latter were enriched for *Aliivibrio* sp. Differences not only in the relative composition but also in the absolute microbial abundance were found between healthy and sick fish, with the sick ones being characterized by higher relative microbial biomasses. This corresponds with the increase in *Aliivibrio* sp. relative abundance and suggests that *Aliivibrio* sp. might be the driver of the observed increase in microbial biomass in the diseased fish. These observations highlight a correlation between the identified *Aliivibrio* sp. and the disease, pointing to a pathogenic or an opportunistic nature of this strain, as also observed with *Vibrio tapetis* on the skin. *Aliivibrio* is a genus that includes known salmon pathogenic strains. The sequence identified in this study matched sequences of two *Aliivibrio* species with 100% identity, not allowing the identification of the exact species. The two hits which showed identical sequences came from two closely related *Aliivibrio* species: *A. logei* and *A. salmonicida*. The latter is a well-known pathogenic species responsible for the cold water vibriosis disease in salmonids. Interestingly, the studied salmon were vaccinated against *A. salmonicida*, therefore the fish are expected to mount an immune response against the pathogen. The fact that *Aliivibrio* sp. is capable of escaping the host's immune control might be a consequence of the compromised health condition of the fish affected by the external *T. dicentrarchi* infection. In these conditions a sick fish may fail to mount an efficient immunological response against *Aliivibrio* sp. These observations suggest that the external infection favors the expansion of other opportunistic species inducing a systemic dysbiosis of the entire gut microbiota community, potentially predisposing the fish to further infections and compromising its fitness.

While the formalin treatment might be useful, and necessary, to treat the external skin infection, it seems not to be beneficial for the gut microbiota community. Samples after treatment presented higher *Aliivibrio* sp. relative abundances, which might be either the result of a failure in containing *Aliivibrio* sp. expansion by the treatment itself, or the consequence of a negative disruptive effect exerted by the treatment on the core gut microbiota. Specifically, in this case, formalin treatment may have had a larger impact in inhibiting growth and abundance of *Mycoplasma* sp. compared to the presumed opportunistic/pathogenic *Aliivibrio* sp. Using Ct values as a proxy for the microbial biomass, and by comparing them before and after treatment, it was possible to show that there was no visible formalin-induced reduction in the gut microbial concentrations. This observation leads to the conclusion that the *Aliivibrio* sp. expansion observed after treatment might not be driven by the treatment itself but just be the result of the progression of the dysbiosis in the salmon gut. This implies that even if formalin is an

effective treatment for external parasitosis, it does not avoid the expansion of other opportunistic/pathogenic strains in the gut whose growth has been presumably primed by the initial external infection, suggesting that other means to actively restore a healthy gut microbiota after formalin treatments may be warranted to avoid the presumably negative effects observed here.

The compromised gut microbiome established during the disease might negatively affect the subsequent health status of the fish by making it more prone to develop further infections or by compromising its growth performance. Given the importance of these two aspects in the aquaculture industry production, it would be advisable to consider strategies, such as probiotic administration, aimed at re-establishing a healthy gut microbiome after formalin treatments.

Compositional and beta diversity results highlighted that the healthy salmon gut microbiota before formalin treatment was almost exclusively characterized by the unknown *Mycoplasma* genus in contrast to what was observed for *Aliivibrio* sp. The Mycoplasmataceae are members of the phylum Tenericutes, class Mollicutes [91]. They are characterized by small-genomes and the absence of a cell-wall [91]. Interestingly they are found in a wide range of habitats but each strain seems specifically adapted to a particular host environment, probably as a consequence of their reduced genome size, which likely constrains their possible ecological niches [91]. This characteristic also makes the Mycoplasmataceae organisms difficult to grow on conventional media. As consequence, the identification of *Mycoplasma* spp. in salmonids and other fish gut has not been possible using culture-based methods. Since the introduction of culture-free methods for microbiome investigations, such as shotgun metagenomics and target-gene amplicon sequencing, *Mycoplasma* species have been more often reported in salmonid gut samples, including commercially relevant species such as Atlantic salmon [26], [89], [92]–[95], Chinook salmon [85], [96], and Rainbow trout [97]–[101], often accounting for the majority of the sequenced reads. Specifically, it has been observed that *Mycoplasma* spp. relative abundances increase during the development [102], and after exposure to salt water, characterizing the microbiome of marine adult salmon [89]. In light of these facts, there is a growing interest regarding this microorganism and the roles that it might play in the fish gut.

The classification of *Mycoplasma* species is not a trivial issue. It has been observed that the *Mycoplasma* genus is in fact a polyphyletic group including species known to be metabolically diverse, with all the species falling into one order (Mycoplasmatales) and one family (Mycoplasmataceae). To address *Mycoplasma* genus polyphyly issue, Gupta and colleagues [91], according to phylogenetic results, have recently proposed the creation of a new order (Mycoplasmoidmales), two new families (Mycoplasmoidaceae fam. nov. and Metamycoplasmataceae fam. nov.) and five new genera. A phylogenetic analysis identified the *Mycoplasma* sp. observed in our study as part of a new, yet undescribed, genus, more closely related with the newly proposed *Malacoplasma* genus [91]. The 16S rRNA gene sequence of the *Mycoplasma* sp. identified in this study clustered with those of other *Mycoplasma* spp. identified in the gut of other fish, constituting, according to our 16S rRNA gene based phylogenetic analysis, a new undescribed genus specifically found in fish intestines. This new *Mycoplasma* genus might be the result of a long-established symbiosis, in which the microorganisms

have evolved to specifically adapt to the fish gut environment [88]. The absence of the microorganism in the surrounding waters observed by previous studies [103], as well as their small genomes, supports this hypothesis.

While some *Mycoplasma* species are known pathogens in humans and other vertebrates where they cause chronic infections [91], the strains found in fish gut have so far not been associated with any negative fitness effects on the host.

In the present study, *Mycoplasma* sp. showed a negative correlation with *Aliivibrio* sp. and characterized the gut microbiota of healthy salmon. Previous studies have reported a negative correlation between *Mycoplasma* and pathogenic bacteria like *Flavobacterium psychrophilum* [97], and genera including potentially pathogenic species such as *Aeromonas* spp. [101] and *Vibrio* spp. [96]. We also found a positive correlation of *Mycoplasma* sp. relative abundance with fish weight (Fig. 4) and condition factor K. Conversely, *Aliivibrio* sp. showed a negative correlation with fish weight (Fig. 4) and condition factor K. Salmon with high *Mycoplasma* sp. relative abundances (> 80% of the sample reads) were statistically larger than fish with high *Aliivibrio* sp. relative abundances (> 80% of the sample reads), pointing to a possible positive effect of *Mycoplasma* sp. in maintaining a healthy gut microbiota.

This study and previous ones have highlighted a potential beneficial role of *Mycoplasma* spp. in preventing disease and enhancing fish growth [97], [101], however the underlying molecular mechanism remains elusive and the absence of a complete genome sequence prevents the possibility to deduce the functional potential of this new *Mycoplasma* genus. Similarly, it is also difficult to infer potential functions of the newly discovered *Mycoplasma* genus relying on its closest relatives with sequenced genomes, as these are the well-known mammalian pathogens *M. penetrans* [104] (16S rRNA gene V3-V4 region sequence similarity of 93.26%) and *M. iowae* [105] (16S rRNA gene V3-V4 region sequence similarity of 91.20%). Whether this *Mycoplasma* genus is capable of conferring some degree of protection from pathogenic infections or enhance fish growth needs further investigations. Multi-omics approaches that include metagenomic assembled genomes and untargeted metabolomics screenings might help to elucidate the genomic feature of this bacteria and the possible functional interactions with the host [107].

Conclusion And Perspective

The present study has shown how an external bacterial infection can cause a systemic shift, favoring the expansion of an opportunistic strain presumably causing dysbiosis of the gut microbiota. This observation is relevant when it comes to treatment optimization, which may include strategies to restore a healthy microbiota profile after infection treatment.

Our results identify the gut microbiota of healthy farmed salmon as dominated by an unknown *Mycoplasma* genus which might be involved in pathogen resistance and/or enhanced growth performances, pointing to a potential mutualistic symbiosis with its host. Further studies are needed to elucidate the role of this microorganism in the fish gut and its putatively positive effects. Here, we

suggest the possibility of utilizing *Mycoplasma* sp. as a biomarker to monitor the health status of farmed salmonids in real-time, possibly through non-invasive sampling procedures. The non-invasive sampling of feces has been shown to provide useful information on the fish gut microbiomes [106] and can hence be implemented for such monitoring strategies. If we assume our results represent a general pattern, then temporal monitoring of the relative abundance of the *Mycoplasma* sp. can be used to detect possible pathogen infections earlier than using e.g. visual identification of external skin ulcers. Such faster diagnostics could allow more timely treatment of the fish before severe phenotypic traits develop, substantially reducing disease associated production losses.

Declarations

Ethics approval and consent to participate

The experimental procedures used in this study were approved by the Norwegian Food Safety Authority. The trial was done under «Forskrift om bruk av dyr i forsøk» 2.F. as a feed trial and was not specifically applied to mattilsynet pre-trial. Use of treatment (Compassionate use) was applied and approved pre-treatment. (https://lovdata.no/dokument/SF/forskrift/2015-06-18-761)

Consent for publication

Not applicable.

Availability of data and materials

Data will be available upon publication on Sequence Read Archive (SRA) at DOI xxxxxx. Scripts and data used for the analysis can be found in the Github repository: https://github.com/DavideBozzi/Bozzi_et_al_2020_analysis.

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Authors' contributions

MTL and MTPG were responsible for the study design. MTL, JAR and KN performed the sampling. DB, in collaboration with JAR, have performed all the laboratory procedures and bioinformatic analysis of the data. CC developed the DNA extraction protocol used in this study. HS and KN were in charge of the experimental design, salmon husbandry and performance of the experimental trial including the formalin treatment. DB drafted the manuscript and all authors read and approved the final version.

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Figures

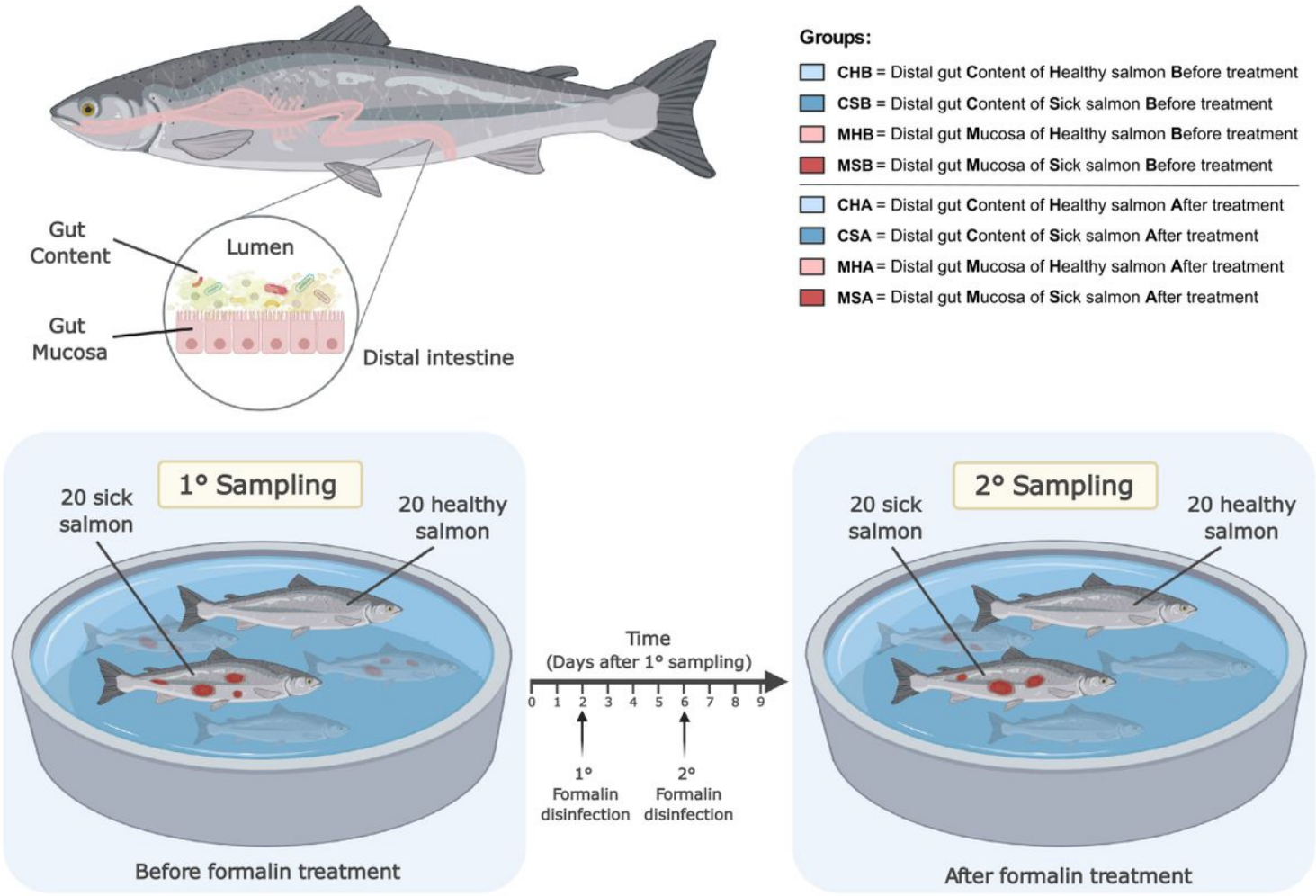

Figure 1

Experimental design and types of samples collected. A total of 80 salmon have been sampled, 40 before and 40 after formalin treatment, in both cases, 20 healthy and 20 sick fish were selected (bottom). Two different types of samples, distal gut content and distal gut mucosa, have been collected for each fish (top-left panel), for a total of 160 samples. According to the experimental design for samples collection, eight groups of 20 samples can be recognized as described in the top-right table: CHB = Distal gut Content of Healthy salmon Before treatment, CSB = Distal gut Content of Sick salmon Before treatment, MHB = Distal gut Mucosa of Healthy salmon Before treatment, MSB = Distal gut Mucosa of Sick salmon Before treatment, CHA = Distal gut Content of Healthy salmon After treatment, CSA = Distal gut Content of Sick salmon After treatment, MHA = Distal gut Mucosa of Healthy salmon After treatment, MSA = Distal gut Mucosa of Sick salmon After treatment. (Created with BioRender.com).

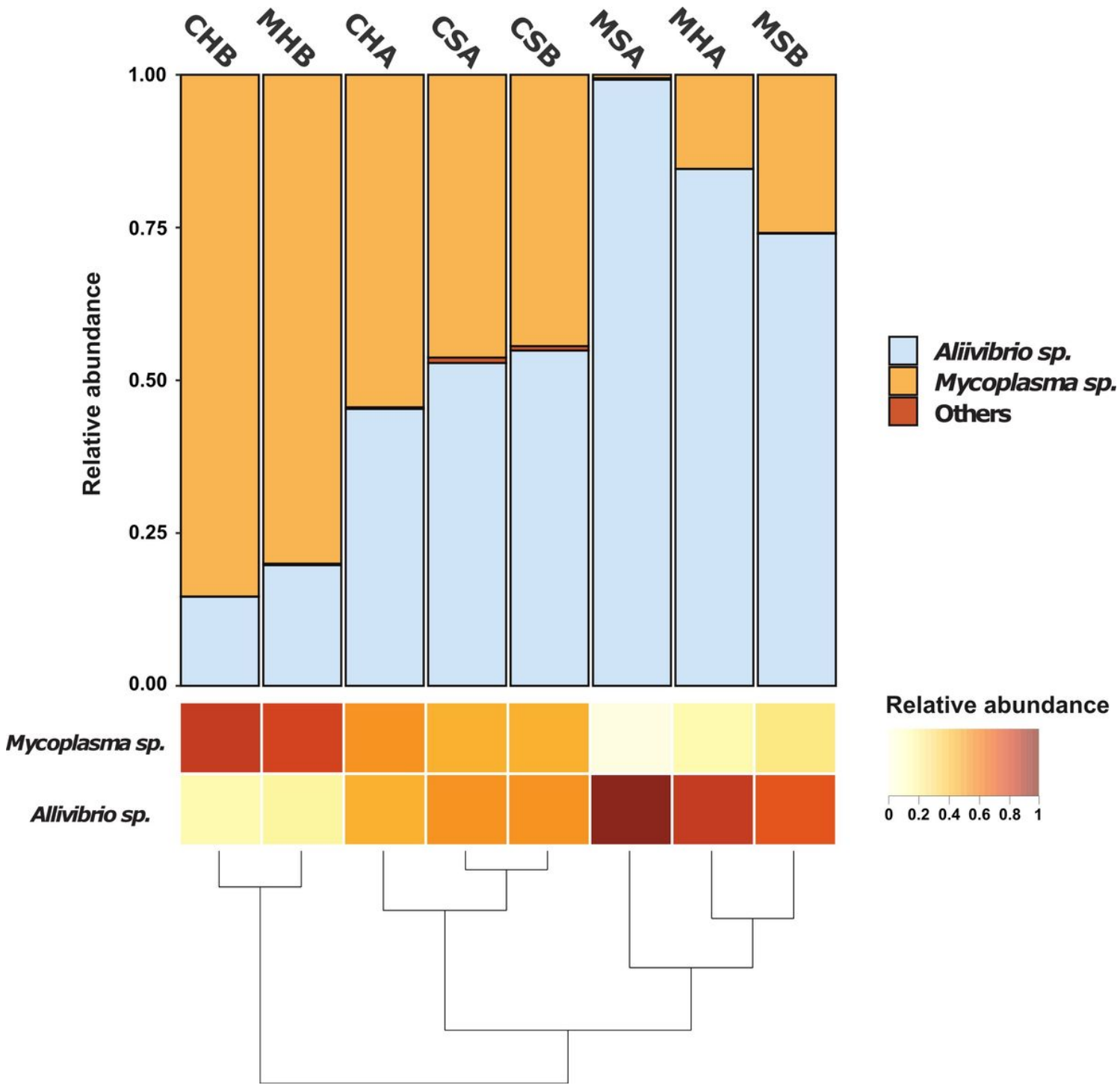

Figure 2

Microbial composition of the investigated groups and groups beta diversity. Barplots depicting the microbial composition of investigated groups (see Fig.1 for definition) shows that they are dominated in the composition by two OTUs: Aliivibrio sp. and Mycoplasma sp. Specific shifts in the relative abundance of the two highly abundant OTUs across groups are visualized. Clustering based on beta diversity (represented as a dendrogram) is mainly determined by the relative abundance of the two dominating OTUs as shown by the heatmap.

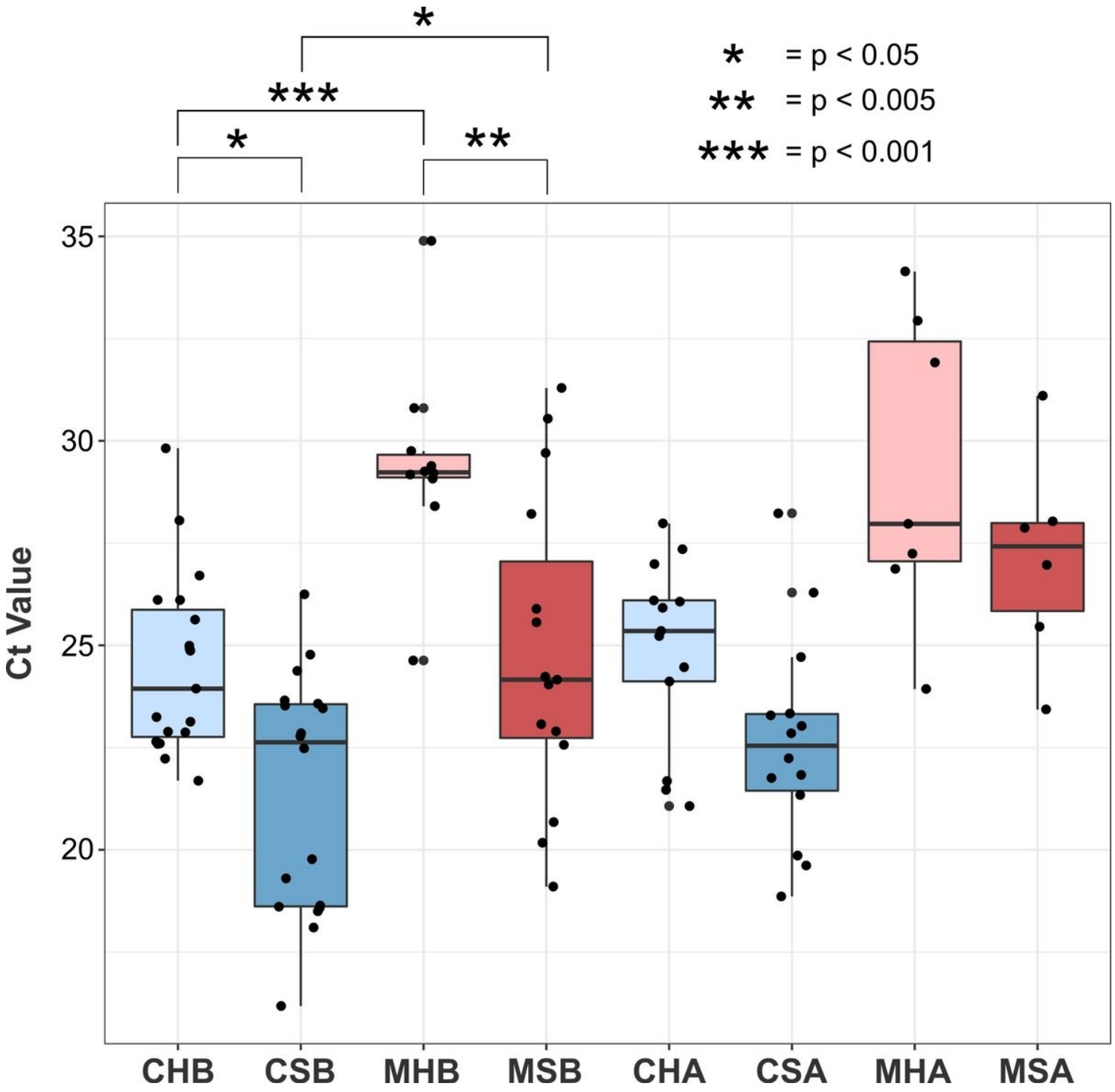

Figure 3

Ct values comparison across groups of samples. The boxplot shows the qPCR Ct values for all sample groups. Groups of samples before formalin treatment (B) are on the left side of the plot, while those after treatment (A) are on the right. Gut content samples are colored in blue while gut mucosa samples in red. Color intensity discriminates between healthy (light) and sick (darker). Differences in the mean Ct values across groups can be seen when comparing groups of healthy fish with their sick counterpart (e.g. CHB vs CSB) and when distal gut content groups with their distal gut mucosa counterpart (e.g. CHB vs MHB). Sick fish present lower Ct values than the healthy ones indicating an increase in the total microbial

biomass in relation to disease progression, and gut mucosa samples harbour lower microbial biomasses than the gut content. No difference in the Ct values could be detected in response to formalin treatment indicating a negligible direct effect on the gut microbial load. Statistically significant differences in the mean Ct value between groups are highlighted for biologically relevant comparisons (see Additional File 10 for all the comparisons p-values).

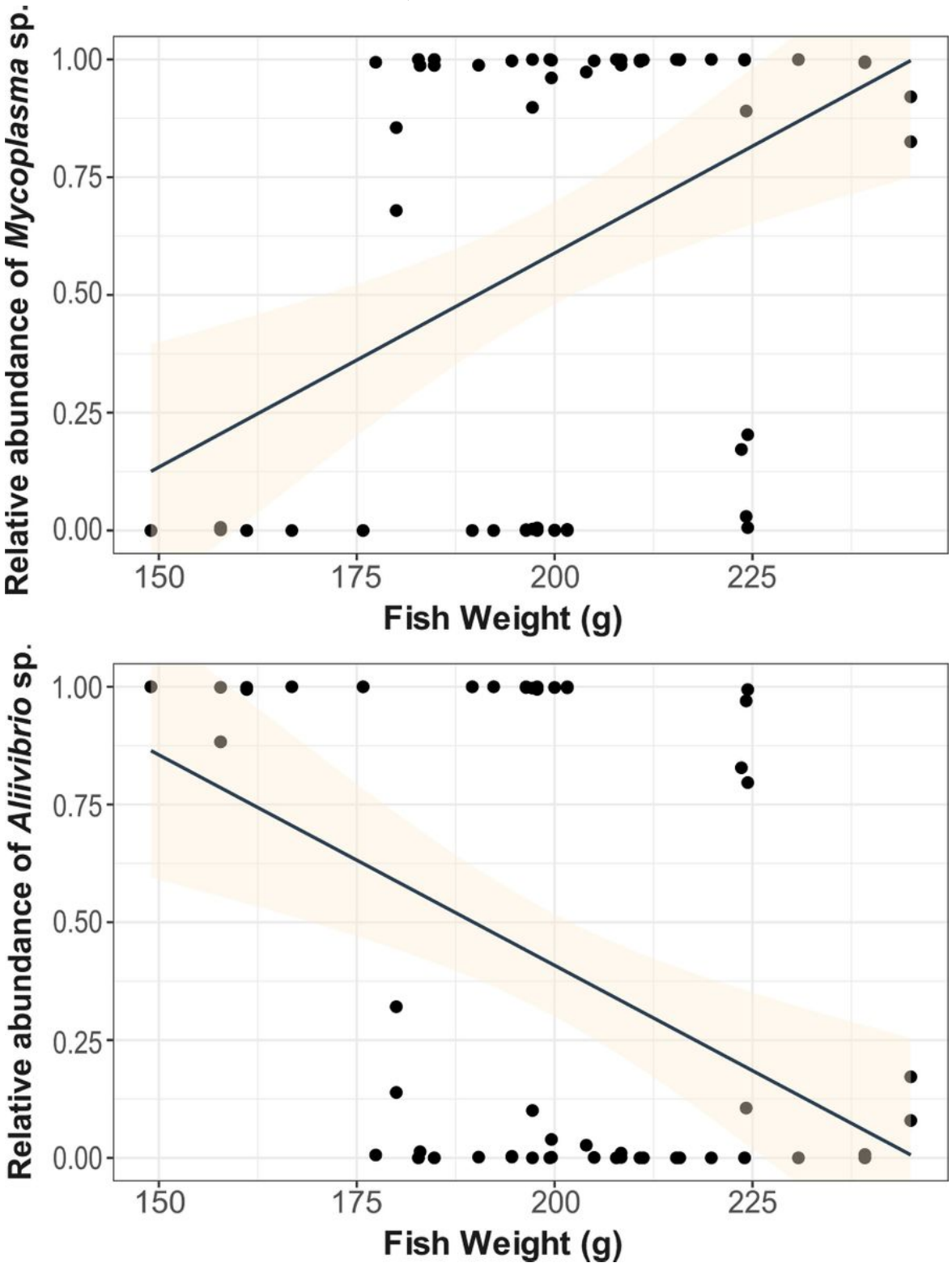

Figure 4

Spearman's rank correlation between OTUs relative abundance and fish weight. A positive correlation (Spearman’s R = 0.43, p < 0.001) was found when comparing Mycoplasma sp. relative abundance and fish weight (top). Vice versa, a negative correlation (Spearman’s R = -0.44, p < 0.001) was observed when comparing Aliivibrio sp. relative abundance with fish weight (bottom).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile10Ctvaluescomparisonp.values.csv
- AdditionalFile9Samplesalphadiversity.csv
- AdditionalFile8OTUtaxonomy.txt
- AdditionalFile7UnrarefiedOTUtable.csv
- AdditionalFile6FastQCPostTrimreport.txt
- AdditionalFile5Ctvalues.csv
- AdditionalFile4PCRinfofile.csv
- AdditionalFile3DNAextractionprotocol.pdf
- AdditionalFile2Metadata.csv
- AdditionalFile1SupplementaryFigures.docx