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Characterization of mid-intestinal microbiota of farmed Chinook salmon using 16S rRNA gene metabarcoding

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Abstract: With the growing importance of aquaculture worldwide, characterization of the microbiota of high-value aquaculture species and identification of their shifts induced by changes in fish physiology or nutrition is of special interest. Here we report the first 16S rRNA gene metabarcoding survey of the mid-intestinal bacteria of Chinook salmon (*Oncorhynchus tshawytscha*), an economically important aquacultured species. The microbiota of 30 farmed Chinook salmon from a single cohort was surveyed using metabarcode profiling of the V3-V4 hypervariable region of the bacterial 16S rRNA gene. Seawater, feed and mid-intestinal samples and controls were sequenced in quadruplicate to assess both biological and technical variation in the microbial profiles. Over 1000 operational taxonomic units were identified within the cohort, providing a first glimpse into the mid-intestinal microbiota of farmed Chinook salmon. The taxonomic distribution of the salmon microbiota was reasonably stable, with around two thirds of individuals dominated by members of the family Vibrionaceae. We anticipate that the workflow presented in this paper could be applied in other aquacultured fish species to capture variation or dysbiosis occurring as a result of changes in feed, health or environmental conditions.

Keywords: farmed salmon; mid-intestinal microbiota; partial 16S rRNA gene sequencing

Abbreviations: non-metric multidimensional scaling (NMDS); operational taxonomic unit (OTU); permutational multivariate analysis of variance (PERMANOVA)

INTRODUCTION

The digestive tracts of all vertebrates harbor complex assemblages of microorganisms (microbial communities), collectively referred to as intestinal microbiota. The intestinal microbiota is an area of research interest universally applicable to Animalia, but the majority of studies on intestinal microbiota composition and function in vertebrates have been conducted in mammals. Comparatively little is known about the fish intestinal microbiota and its response to changing environmental conditions [1], despite the fact that fish represent roughly half of all living vertebrate species [2] and are of global economic significance.

The microbial community harbored in fish intestines influences host physiology and is therefore of relevance to aquaculture. Gnotobiotic and conven-

tional studies indicate the involvement of the intestinal microbiota in fish nutrition, development of intestinal epithelium, immunity and disease [3]. During the past few decades, substantial research has been carried out to characterize the intestinal microbiota in a wide range of fish species, focusing primarily on model organisms (i.e. zebrafish) and species relevant to aquaculture. As studies of fish intestinal microbial diversity have moved away from culture- and microscopy-based observations to the culture-independent molecular techniques, it has become clear that the intestinal microbiota of fish is more variable than previously realized. High throughput partial 16S rRNA gene sequencing has been increasingly employed to investigate changes in the structure of fish intestinal microbial community caused by diet (including probiotics), starvation, pathogens, different lifestyles and water temperature [1,4,5].

Fish intestines harbor a combination of resident (autochthonous) microbiota, attached to the intestinal mucosa, and non-resident (allochthonous) microbiota, comprised of microbes appearing transiently and/or associated with digesta [6,7]. The composition of fish intestinal microbiota and species richness varies with life stage, diet and environment [3,8] and differs between marine and freshwater species [9]. Fish intestinal microbiota also varies between individuals, across the length of the gastrointestinal tract, and between intestinal content and mucosal surfaces [1,5,10-12]. Interaction between time of sampling and diet is strongly related to the observed community structure [13]. Nonetheless, phylogenetic and statistical analyses of 16S rRNA gene libraries suggest the presence of persistent members of autochthonous teleost intestinal communities, the 'core' microbiota that remains stable despite changing factors [4,14]. 'Core' microbiota has been observed not only within a single fish species, but also may be shared between broad ranges of fish species [5,15,16].

Most fish intestinal microbial communities investigated to date comprise microbes from the phyla Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Planctomycetes, Proteobacteria and Tenericutes [1,3,17]. The genera *Aeromonas* and *Pseudomonas* (Proteobacteria) and phylum Bacteroidetes dominate freshwater fish bacterial communities, while the genera *Vibrio*, *Pseudomonas* and *Alteromonas* (Proteobacteria) are reported to predominate the intestines of marine fish [1,3,9]. Proteobacteria and Firmicutes are the most reported phyla in the salmonid intestines [7], although current knowledge of the bacterial diversity in the salmon gastrointestinal tract is largely based on classical culturing techniques. Dominant culturable bacteria isolated from intestines of salmonid fish species include *Vibrio*, *Aliivibrio*, *Photobacterium*, *Lactobacillus*, *Lactococcus*, *Flavobacterium*, *Pseudomonas* and assorted *Enterobacteriaceae*. Recent sequencing-based studies have focused on salmonid species most prevalent in aquaculture – especially Atlantic salmon (*Salmo salar*) [6,19-24], Coho salmon (*Oncorhynchus kisutch*) [24] and rainbow trout (*Oncorhynchus mykiss*) [11,26-30]. To our knowledge, no such studies have been published on Chinook salmon (*Oncorhynchus tshawytscha*), also known as king salmon, a species that is farmed in commercial quantities mainly in New Zealand and Chile.

The aim of this study was to identify the mid-intestinal microbiota of farmed Chinook salmon and assess the effects of biological variation between fish mid-intestinal microbiota and technical variation between and within sequencing run. For this reason, our experimental design included duplication of the library indexing (to assess intra-run variation) and sequencing (to assess run-to-run variation) steps.

MATERIALS AND METHODS

Ethics statement

The fish examined in this research were obtained from the commercial salmon farm (NZ King Salmon farm) after normal commercial harvest and prior to gutting and gilling. All fish were harvested according to standard NZ King Salmon operational practice [30]. Under New Zealand's Animal Welfare Act 1999, dissections on carcass material do not require approval by the animal ethics committee.

Fish management

Chinook salmon used in this experiment were obtained from a commercial NZ King Salmon (NZKS) farm (Ruakaka Bay Farm in Queen Charlotte Sounds,

New Zealand) during a standard harvest operation. All sampled fish were female (which is a standard practice in New Zealand salmon farming [31]), approximately 22 months old, and belonged to a single cohort. Individuals were reared in sea pens (20 m x 20 m x 15 m) using standard farm management practice [30]. The fish were fed a Quinnat Plus 2200 commercial diet (BioMar, Denmark), delivered to the sea pens via a mobile hopper twice a day using a satiation feeding approach, until the harvest. No antibiotics, probiotics, antifungals, antivirals or antiparasitics were used during the rearing of the salmon.

Sample collection and processing

Samples were collected in January 2015 (mid-summer) at the salmon farm from 30 apparently healthy Chinook salmon. The fish were harvested at an average weight of 3.6 kg using standard NZKS operational practice [30] and processed on the barge immediately after slaughter. The fish were dissected, and the length and the appearance of the mid-intestine, measured from the last pyloric caecae to the start of the distal intestine, was recorded. For each fish, a single 1-cm section of the mid-intestine, including its content, was collected from the first half of the mid-intestines (roughly 2 cm past the pyloric caeca) using sterile instruments and placed into sterile tubes containing 5 mL RNA solution (Ambion, USA). Several feed pellets from the spinner supplying the relevant pen and 5 mL of seawater from the surface of the pen were also collected into sterile tubes containing 5 mL RNAlater solution. A sterile tube containing 5 mL RNAlater solution was handled identically to the rest of the samples, including being carried to the sampling site (barge) and briefly exposed to the air (negative control). The samples were transported on ice packs to the laboratory and stored at 4°C for two weeks prior to DNA extraction.

For each fish, the mid-intestinal section was removed aseptically from the RNA and opened longitudinally to release digesta using sterile forceps and scalpel, and the appearance of the intestinal content was recorded. The opened mid-intestinal tissue section was "washed" in 3 mL of RNAlater suspension in which it was stored by vigorous vortexing at maximum speed for 30 s to release and homogenize intestinal content. The washed intestinal tissue was then aseptically removed, and the remaining homogenized mid-intestinal sample (with intestinal content) was split into two 1.5-mL aliquots. One aliquot was used directly for the DNA extraction. For the other aliquot, prior to DNA extraction, material gently scraped from the mucosal surface of removed intestinal tissue was added to the sample to increase the likelihood of collecting bacterial cells adherent to the gut epithelium or trapped in the mucus layer. For each fish, DNA was extracted from two 1.5-mL aliquots of the salmon mid-intestinal samples (one with and one without the addition of scraped mucosal material). Approximately 100-200 mg of feed pellets were crushed aseptically using a mortar and pestle and homogenized in 1.5 mL of RNAlater by vortexing. The seawater sample was not processed prior to DNA extraction.

DNA extractions

DNA was extracted from two 1.5-mL aliquots of the salmon mid-intestinal sample/RNAlater suspensions (one with and one without the addition of scraped mucosal material), as well as from 1.5 mL of seawater/RNAlater suspension and 1.5 mL of feed/RNAlater suspension. Because of several possible sources of bacterial DNA contamination during sampling and DNA extraction [32], a DNA extraction was also performed from 1.5 mL of RNAlater carried during the sampling trip (negative control, to account for bacterial DNA being introduced from the DNA extraction and sample handling). DNA was extracted with a NucleoSpin Soil kit (Macherey-Nagel, Germany) using an adapted manufacturer's protocol, and DNA yield, purity and integrity, as well as the presence of bacterial and host DNA in mid-intestinal samples and controls, were assessed as described in the Supplementary Material (subheading: 'DNA extractions').

16S rRNA amplicon library preparation and sequencing

DNA extracted from two salmon mid-intestinal samples (one with and one without the addition of scraped mucosal material) were pooled for the amplification of the V3-V4 region of 16S rRNA gene. A series of template dilutions (1-, 2-, 5- and 10-fold dilutions) were tested for each sample and the dilution that produced

the strongest 16S amplicon band, judged by agarose gel electrophoresis, was chosen for sequencing. For seawater and feed samples, the RNAlater, the no-template control (molecular water) and genomic DNA from the mock microbial community HM-782D [33], 16S amplicons were produced without template dilution. The detailed protocol on the preparation of sequencing library can be found in Supplementary Material (subheading: '16S rRNA amplicon library preparation').

Samples were normalized where possible to the equivalent concentration of 10 ng/μL and sent to the sequencing provider New Zealand Genomics Limited (NZGL) for indexing and sequencing. Indexing was performed by NZGL using the Nextera XT Index Kit (Illumina, USA) in duplicate for each sample, with two distinct dual index pairs used for each sample to assess the effect of index and intra-run variability. This dual indexing of the initial library of 35 samples (30 fish samples, seawater and feed samples, and 3 controls) resulted in a final sequencing library of 70 samples. All samples were pooled without normalization and sequenced twice using the Illumina MiSeq system with v3 reagents to produce 2x300 bp reads. This platform and this particular chemistry were chosen, among available options at the time of sequencing, because they offered the most favorable combination of number of reads and their length at lowest cost. Two separate 600-cycle 65-h runs were performed using two MiSeq instruments to determine 'run-to-run' variation (technical replicates). For each of 30 fish and 5 controls, samples were indexed twice and each of these two sample subsets were sequenced twice, in two separate sequencing runs. The reads were processed and assigned to OTUs using the QIIME software package, version 1.8.0 [34], as described in the Supplementary Material (subheading: 'Sequence analysis').

RESULTS

Sample collection and preparation of 16S rRNA amplicon library

Sampled salmon mid-intestines ranged in length from 9 to 15 cm. The majority of samples (26) had a normal appearance, while the remaining 4 had lesions visible on the inner intestinal lining. Although the sampled salmon had been fed as per usual farm protocol until the harvest, only 8 contained visible feed content. Quality checks of the DNA samples extracted from salmon mid-intestinal samples, seawater, feed and RNAlater (control), as well as of the 16S rRNA amplicons, are discussed in Supplementary Material (subheading: '16S rRNA amplicon library').

Sequencing metrics and analysis

Sequencing yielded a total of 18 million 16S rRNA gene amplicon sequences, combined over both sequencing runs. Approximately 10.2 million and 7.9 million of reads were assigned to an index in the first and the second sequencing run. Raw sequences from Chinook salmon mid-intestinal microbiome 16S rRNA gene metabarcoding survey generated on the Illumina platform are publicly available through the NCBI Sequence Read Archive (SRA) database. SRA accession is SRP134829 (https://www.ncbi.nlm.nih.gov/sra/SRP134829).

Following read pair joining, removal of short sequences and stringent quality filtering, sequence numbers were reduced to 750000 sequences spread over 140 samples (Supplementary Table S3). All identified OTUs and their occurrence in each group can be found in the Supplementary File (QIIME_qc30.final.txt). A full list of all OTUs identified during the quality control stages is reported in Supplementary Table S1. The overall sequencing error rate, based on sequencing mock microbial community, was 0.49%. The number of sequences recovered and OTU richness in all the controls (RNAlater, no-template control and mock microbial community) was low, indicating very low levels of contaminating DNA sequences in these controls.

Mid-intestinal microbiota of farmed Chinook salmon

A total of 1308 OTUs were identified within the mid-intestinal microbiota of the 30 salmon. The salmon mid-intestinal microbiota was typically dominated by several abundant bacterial lineages (Fig. 1; Supplementary Table S2). Notably, the mid-intestinal microbiota of the majority of fish was dominated by the family *Vibrionaceae* (Figs. 3 and 4). The other OTUs present in at least 60% of individuals repre-

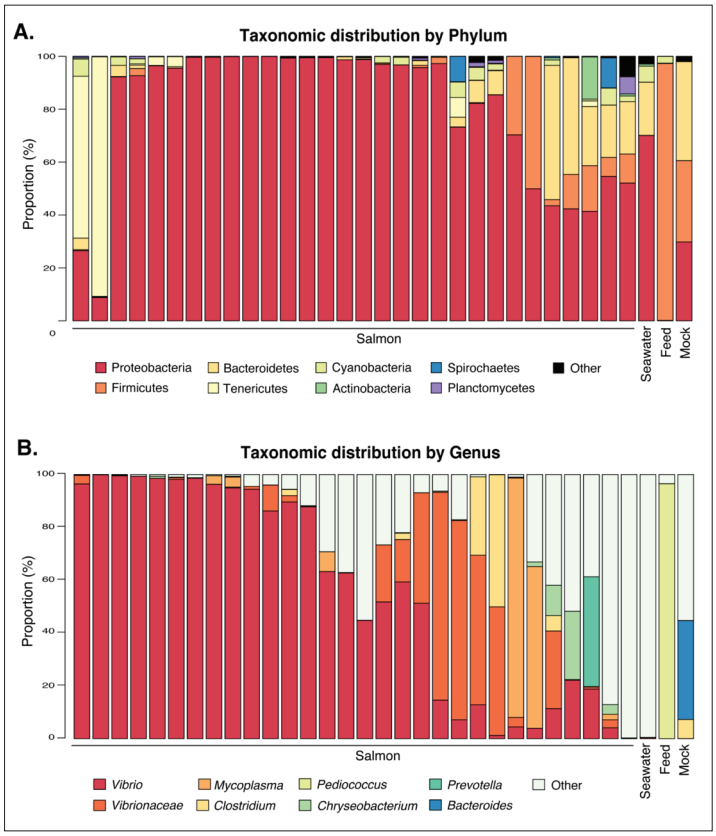

Fig. 1. Phylum (A) and genus (B) level taxonomic distribution of the surveyed microbiomes. Bars report the mean abundance for each individual sample. The top 9 most abundant genera (across all samples) are reported, all others are aggregated into 'Other'.

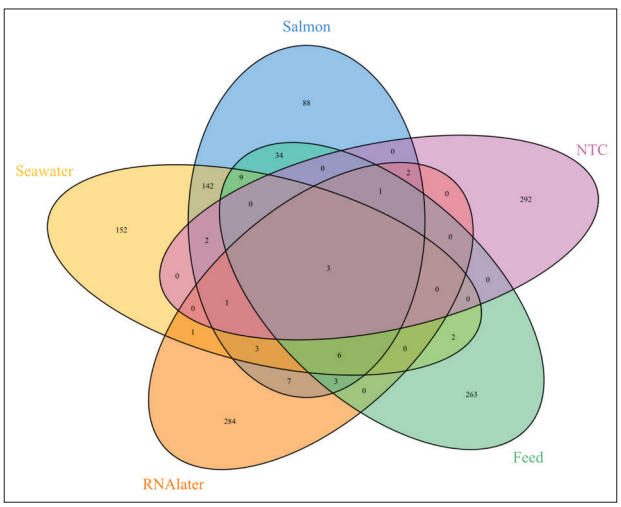

Fig.2. Venn diagram of shared OTUs between salmon and control samples. OTUs (operational taxonomic units) are represented at 97% sequence similarity.

sented the genera *Synechococcus*, *Clostridium*, *Pseudomonas*, *Chryseobacterium*, *Brevundimonas*, *Sphingomonas*, *Paracoccus,* NS5 marine group, NS4 marine group, *Polaribacter*, *Acinetobacter*, *Sulfitobacter*, *Loktanella*, *Photobacterium* and *Pseudoalteromonas*, or, where a specific genus name could not be assigned, the family Rhodobacteraceae and orders Rickettsiales SAR116_clade, Rhizobiales and Oceanospirillales. The taxonomic distribution of the salmon microbiota was conserved between individuals, but notably different between salmon and environmental samples (Figs. 1 and 2).

Analysis of variation

Alpha diversity indices (Chao1 richness estimator and the Shannon and inverse Simpson diversity estimators), calculated from the final OTU table, are presented in the Supplementary Addendum (QIIME_qc30.alpha.txt). Five measures of community structure (Jaccard, unweighted UniFrac, Bray-Curtis, Yue-Clayton theta and weighted UniFrac distances) were computed from the final OTU table. Measures were performed using both the full ('uncorrected') and 'error-corrected' data table. Boxplot visualization of the PERMANOVA data (distribution of fit measurements according to metadata characteristics for all calculated beta diversity measures, reported according to error correction strategy) showed high variability (Fig. 5). High variability in the distribution of fit measurements according to metadata characteristics for all calculated beta diversity measures is due to the aggregation of multiple beta diversity scores using multiple subsampling strategies. For example, unweighted UniFrac typically performed poorly in the individual category (median R2=0.42), while Yue-Clayton provided an excellent fit for data (median R2=0.97). Manual identification and analysis of a subset of the data with highly similar amplicon yields, as determined by densitometry, showed the same pattern as for the full data set (data not shown).

Visualization of this data performed using a NMDS plot of community structure, based on Yue-

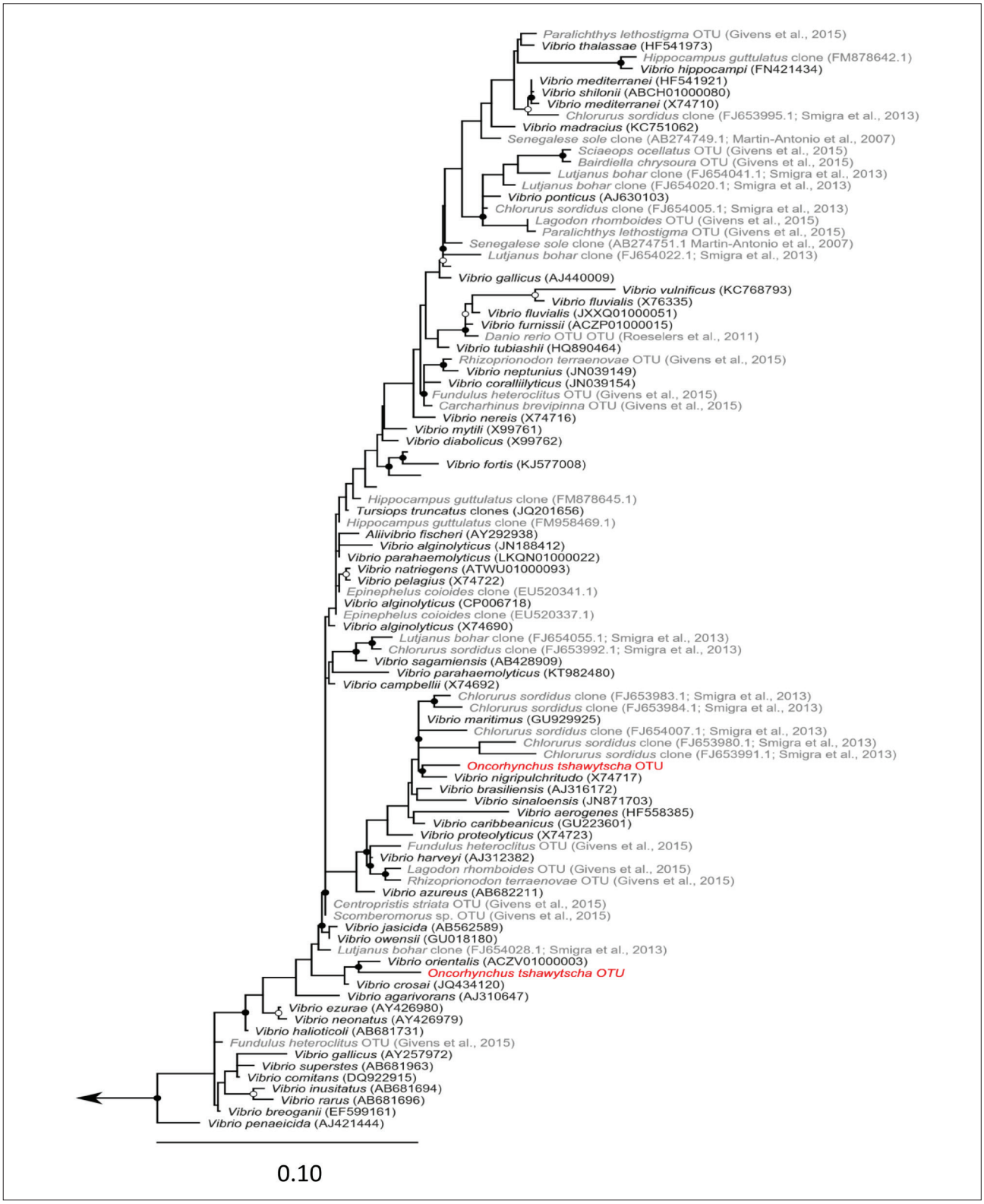

Fig. 3. Phylogenetic analysis of *Vibrio*-like 16S rRNA sequences obtained from marine vertebrates. Sequences in black were obtained from cultivated *Vibrio* isolates, and those in grey from publicly available microbiome surveys. Sequences in red are *Vibrio*-like OTUs from this data set. Dashed lines represent short sequences (<1000 bp) inserted into the fixed tree. Bootstrap support is represented by solid (≥90%) and hollow (≥75%) junctions. Scale bar represents 10% sequence divergence.

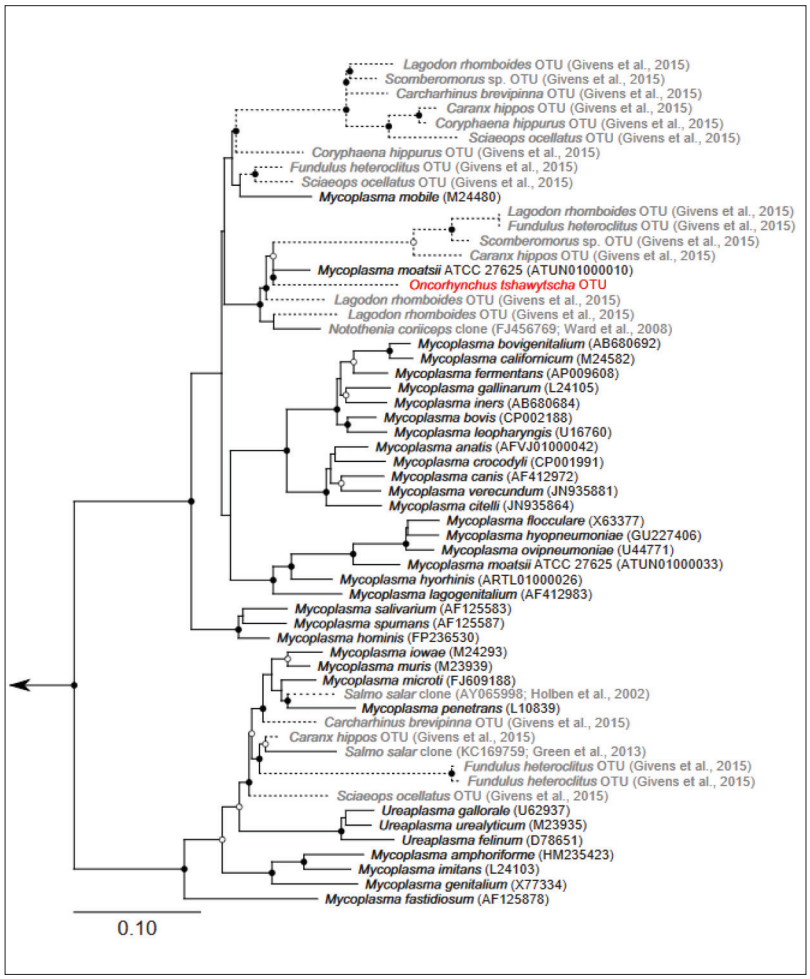

Fig. 4. Phylogenetic analysis of *Mycoplasma*-like 16S rRNA sequences obtained from marine vertebrates. Sequences in black were obtained from cultivated *Mycoplasma* isolates and those in grey from publicly available microbiome surveys. Sequence in red represents the *Mycoplasma*-like OTU from this data set. Dashed lines represent short sequences (<1000 bp) inserted into the fixed tree. Bootstrap support is represented by solid (≥90%) and hollow (≥75%) junctions. Scale bar represents 10% sequence divergence.

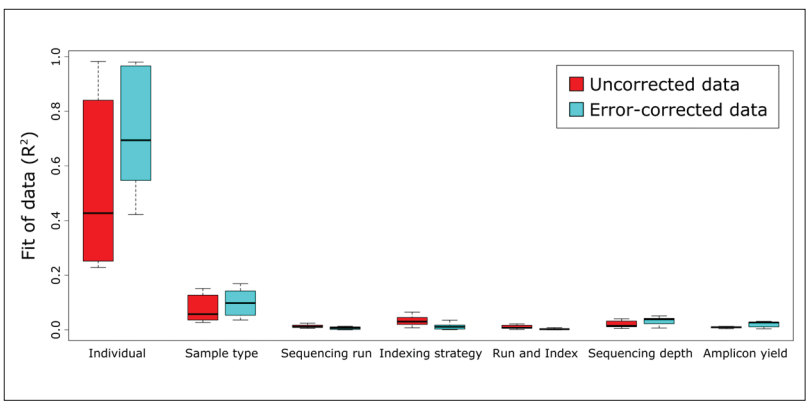

Fig. 5. Average fit of data based on metadata characteristics. Boxplot visualization of the distribution of fit measurements according to metadata characteristics for all calculated beta diversity measures. Data are reported according to error correction (i.e. contaminant removal) strategy, as defined in section 2.4. High variability is due to the aggregation of multiple beta diversity scores using multiple subsampling strategy. For example, unweighted UniFrac typically performed poorly in the Individual category (median R2=0.42), while Yue-Clayton provided an excellent fit for data (median R2=0.97). Nonetheless, it is clear that the majority of variation is attributable to variation between individuals.

Clayton theta distance with no subsampling (Fig. 6), showed that the 4 technical replicates for each sample clustered together, while feed and seawater samples and controls (NTC, RNAlater, mock) were distinct. With the exception of the RNAlater control in the 'uncorrected data', the microbiota present in the additional samples (feed, seawater) did not have a significant effect on the fish intestinal microbiota, although it is likely that these sources act to seed the intestinal microbiota with certain microbial lineages.

A list of all OTUs identified during quality control stages is reported in Supplementary Table S1. Removal of OTUs associated with negative controls or with distributions inversely correlated to amplicon yield [35] improved the fit of the data (Fig. 5).

Diversity and individual variation in salmon mid-intestinal microbiota

The mid-intestinal microbiota of sampled salmon was less diverse than seawater and also contained a lower species richness Supplementary Addendum (QIIME_qc30.alpha.txt). The presence of intestinal digesta at the time of sampling was the only

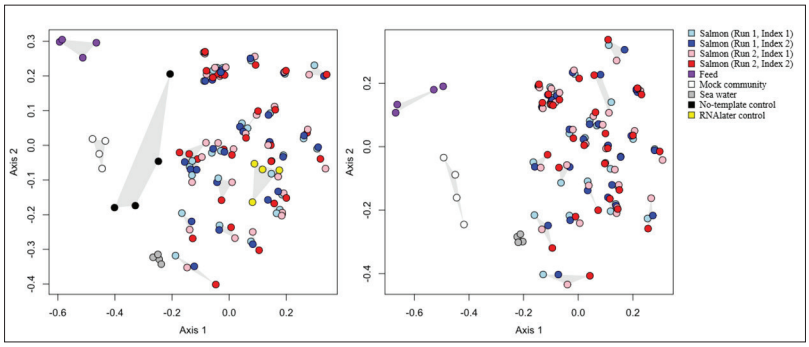

Fig. 6. NMDS plots of community structure. Plots are based on Yue-Clayton theta distance with no subsampling. Shadowing reflects clustering of individual samples. Left: Uncorrected data (stress=0.24, r2=0.95). Right: Error-corrected data (stress=0.23, r2=0.95). Controls (NTC, Mock, Feed, Sea water, RNAlater) cluster separately; the remaining clusters tend to be composed of the 4 technical replicates from an individual fish.

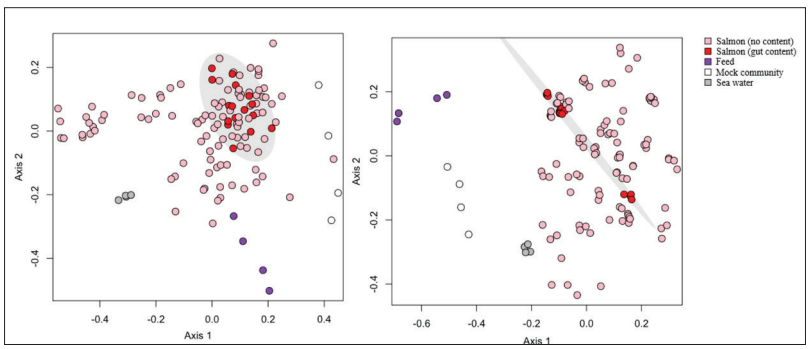

Fig. 7. NMDS plot of salmon gut microbiota with the presence of gut content recorded. Data presents the error-corrected data set without subsampling, and samples are colored based on sample type. Ellipses represent the 95% confidence interval around all samples with recorded gut content. Left: Jaccard distance (stress=0.20, r2=0.96). Right: Yue-Clayton theta distance (stress=0.23, r2=0.95).

parameter with observable effect and although the presence of digesta did not drive a strong separation of salmon samples, there was a tendency for these samples to cluster within the broader salmon data (Fig. 7).

DISCUSSION

Although the sampled salmon had been fed as per usual farm protocol until the harvest, only about one third of sampled salmon had visible feed content in their intestines. Sampling was performed during a mid-summer water temperature spike, and salmon tend to go off feed when water temperatures are high [36]. Following the completion of this study, salmon farms in the region, including the one surveyed, reported elevated fish mortality (>30%) over the sampling period, which was likely associated with this stressor. Although the sampled fish appeared healthy, due to the starvation and temperature variations it is unknown whether the microbial profiles reported here would remain similar for fish without these environmental stressors.

The mid-intestinal microbiota of the majority of fish was dominated by the family *Vibrionaceae*, as has been observed in Atlantic salmon (*Salmo salar*) [23]. Interestingly, two of the 30 sampled individuals had a microbiota dominated by *Mycoplasma*; dominance of *Mycoplasma* spp. has been seen before in wild-caught Atlantic salmon [19]. Phylogenetic inference of OTUs belonging to these lineages demonstrated that these OTUs are closely related to species identified in similar analyses of fish microbiota [16,20,38-41] (Figs. 3 and 4). The observation of *Vibrio* and *Mycoplasma* is consistent with typical fish-associated species and they are unlikely to represent colonization by novel lineages.

When attempting to characterize a "core microbiome" shared among the sampled salmon, it was apparent that at the level of individual OTUs, the microbiota of the fasting fish is quite variable within this single cohort. This is not surprising, as conservation of intestinal microbiota occurs primarily at the level of metabolic function, while the specific bacterial species fulfilling that function within an individual animal can vary significantly [41,42].

A recent study of the effects of starvation on the Asian seabass (*Lates calcarifer*), another farmed carnivorous species, reported a major shift in the intestinal microbiota towards members of the phylum Bacteroidetes, driven by an increase in members of the classes Sphingobacteria and Bacteroidia and a decrease in the Betaproteobacteria [43]. Without a control group of salmon feeding normally, it is im-

possible to determine if a similar effect is occurring in this data, but if so, this shift is not apparent in the core microbiota of the sampled cohort. Members of Bacteroidetes in the salmon only approached the levels of Proteobacteria in 10% of individuals (three fish).

Although the microbiota of the pellet feed was dominated by a strain of *Pediococcus* (Fig. 1; median abundance 96.5%), this organism was almost undetectable in the salmon mid-intestines (<0.01%). *Pediococcus acidilactici* strain MA 18/5M is sold as the probiotic product Bactocell® (Lallemand Inc., Canada) for reducing intestinal inflammation in fish [44], and this product is added to some BioMar feed pellets (BioMar Group, Denmark). The low abundance of this organism in the intestine suggests that the organism is not able to establish in the intestines and quickly declines when salmon are not actively consuming the probiotic.

Importantly to aquaculture of these fish, an OTU matching *Piscirickettsia salmonis* was detected in four individuals. This is consistent with the finding of *P. salmonis*-like bacteria at multiple salmon farms in the Marlborough Sounds [45,46], including the farm sampled during this study. Accurate taxonomic identification from partially sequenced 16S rRNA gene fragments is problematic [47,48], but as *P. salmonis* is a known salmon pathogen [49], this detection demonstrates the applicability of 16S rRNA gene metabarcoding for detection of potential pathogens in asymptomatic individuals and highlights the need to follow up suspicious findings with targeted diagnostic tests.

Under all 5 distance metrics tested, the effects of the biological variables (individual variation, sample type) in the study were profoundly greater than the technical aspects – sequencing run, index and amplicon yield. Although the individual donor and amplicon yield variables are highly confounded, analysis of a data subset with highly similar amplicon yields showed the same pattern as for the full data set. The data demonstrate that the microbiota donor is the strongest contributor towards community structure, and that technical parameters quantified within this study do not significantly influence patterns in community structure.

Overall, it appears that the effects of technical variation, including amplicon yield, indexing and MiSeq run, when each step is performed by the same individual and instrument, are negligible. Contamination by reads from the kits and reagents, however, does appear to have a minor effect and should be accurately quantified and removed from the data prior to analysis. Overall, although the impact of contamination is negligible on the level of gross variation between individuals, it does have an effect. Batch-to-batch variations of kit and reagent microbial contaminant profiles were previously reported [32,50]; therefore we highly recommend running such controls for every individual kit and PCR reagent batch used.

The finding of lower microbial diversity in sampled salmon compared to seawater is expected, as the acquisition and maintenance of fish intestinal microbiota is a complex process. It is driven by both environmental availability of potential microbial colonizers and host physiological pressures in a highly selective gut environment [51,52].

Observed clustering of samples with intestinal content is not unexpected, as the digesta is the primary source of nutrients for the intestinal microbiota, thus affecting the microbial community structure. The apparent lack of similarity between the microbiota structure of the fed salmon mid-intestines and the feed pellets provides evidence that the food source is not contributing significantly to the colonizing intestinal microbiota.

In conclusion, we conducted the first 16S rRNA gene metabarcoding survey of the bacterial intestinal microbiota of farmed Chinook salmon. Over a thousand OTUs were identified within the intestines of a cohort of 30 fish, providing a first glimpse into the mid-intestinal microbiota of this aquacultured species. Our survey was performed during a summer water temperature spike and it is unknown whether the microbial profiles reported here would remain similar for fish without this environmental stressor.

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Conflicts of interest disclosure: The authors declare that there are no financial or non-financial conflict of interest in the publication of this manuscript.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Ciric%20et%20al_Supplementary%20Material_4144.pdf

Supplementary File (QIIME_qc30.final.txt): http://serbiosoc.org.rs/NewUploads/Uploads/Ciric%20et%20al_4144_Supplementary%20File_QIIME_qc30.final.txt.txt