Stable Core Gut Microbiota across the Freshwater-to-Saltwater Transition for Farmed Atlantic Salmon

Knut Rudi,^a Inga Leena Angell,^a Phillip B. Pope,^a Jon Olav Vik,^b Simen Rød Sandve,^b Lars-Gustav Snipen^a

^aFaculty of Chemistry, Biotechnology and Food Science, University of Life Sciences, Ås, Norway

^bFaculty of Biosciences, University of Life Sciences, Ås, Norway

ABSTRACT Gut microbiota associations through habitat transitions are fundamentally important yet poorly understood. One such habitat transition is the migration from freshwater to saltwater for anadromous fish, such as salmon. The aim of the current work was therefore to determine the freshwater-to-saltwater transition impact on the gut microbiota in farmed Atlantic salmon, with dietary interventions resembling freshwater and saltwater diets with respect to fatty acid composition. Using deep 16S rRNA gene sequencing and quantitative PCR, we found that the freshwater-to-saltwater transition had a major association with the microbiota composition and quantity, while diet did not show significant associations with the microbiota. In saltwater there was a 100-fold increase in bacterial quantity, with a relative increase of *Firmicutes* and a relative decrease of both *Actinobacteria* and *Proteobacteria*. Irrespective of an overall shift in microbiota composition from freshwater to saltwater, we identified three core clostridia and one *Lactobacillus*-affiliated phylotype with wide geographic distribution that were highly prevalent and co-occurring. Taken together, our results support the importance of the dominating bacteria in the salmon gut, with the freshwater microbiota being immature. Due to the low number of potentially host-associated bacterial species in the salmon gut, we believe that farmed salmon can represent an important model for future understanding of host-bacterium interactions in aquatic environments.

IMPORTANCE Little is known about factors affecting the interindividual distribution of gut bacteria in aquatic environments. We have shown that there is a core of four highly prevalent and co-occurring bacteria irrespective of feed and freshwater-to-saltwater transition. The potential host interactions of the core bacteria, however, need to be elucidated further.

KEYWORDS 16S rRNA gene, microbiota

Gut bacteria are a key part of both terrestrial and aquatic animal life. However, these contrasting host-associated environments are fundamentally different with respect to dispersal and survival of microorganisms (1). We are starting to understand the dispersal and importance of gut bacteria in terrestrial environments (2), while our knowledge about gut bacteria in aquatic environments is still very limited. In particular, little is known about the effect of environmental factors, such as water salinity, on the interindividual distribution of gut bacteria (3, 4).

For anadromous fish, freshwater-to-saltwater migration represents a major shift in both environmental microbial exposure (3, 4) and nutrient availability—in particular, lipid sources which are low in long-chain polyunsaturated fatty acids (LC-PUFA) in freshwater and high in saltwater (5). It has recently been shown that the freshwater-to-saltwater transition has a major impact on the skin mucosal microbiota for the anadromous Atlantic salmon (*Salmo salar*) (6). However, current studies on the gut microbiota of farmed Atlantic salmon have not yet addressed the impact of this

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Address correspondence to Knut Rudi, knut.rudi@nmbu.no.

FIG 1 Composition and distribution of the microbiota in saltwater and freshwater. (A) Distribution in freshwater and saltwater for dominant bacterial phyla. (B) The distributions across treatments, freshwater, and saltwater were determined by PCA. VO, vegetable oil; MA, marine oil.

transition (7–14) or how the environmental exposure and/or nutrient availability affects the composition and interindividual distribution of the gut microbiota.

Accordingly, the aim of our work was to investigate the effect of freshwater-to-saltwater transition under two contrasting diets that have a freshwater-type lipid composition low in LC-PUFA and a high LC-PUFA marine-like lipid composition. In order to explore the microbiota, we used a combination of quantitative PCR (qPCR) and 16S rRNA gene deep sequencing.

We present results showing a distinct shift in overall microbiota potentially associated with the freshwater-to-saltwater transition, while there were four co-occurring core bacteria with wide geographic dispersal exerting stability across this transition.

RESULTS

Characterization of microbiota composition and distribution. By deep sequencing we obtained a total of 13,752,775 paired-end merged 16S rRNA gene sequences passing the quality filter. For these we identified a total of 1,179 prokaryote operational taxonomic units (OTUs) belonging to 20 phyla, with 5 phyla constituting >90% of the microbiota.

The overall microbiota composition differed clearly between freshwater and saltwater, as seen in Fig. 1, and from the analysis of variance (ANOVA), where this effect was very clear ($P < 10^{-10}$). There were 413 OTUs that were significantly affected by the freshwater-to-saltwater transition ($P < 0.05$; false-discovery rate [FDR] corrected by the Benjamin and Hochberg approach), for which a majority (76.5%) showed decreases in saltwater. The frequency of OTUs with high relative quantity, on the other hand, increased in saltwater (see Fig. S1 in the supplemental material). The main taxonomic shift from freshwater to saltwater was a decrease in both *Actinobacteria* (median, 4.4% versus 3.5%; $P < 0.0005$) and *Proteobacteria* (median, 7.6% versus 5.4%; $P = 0.002$), while *Firmicutes* showed a major increase (median, 48.5% versus 72.7%; $P < 0.0005$). Both the classes *Clostridia* (median, 33.6% versus 50.2%; $P < 0.0005$) and *Bacilli* (median, 14.9% versus 20.5%; $P < 0.0005$) increased. *Alphaproteobacteria* increased (median, 0.7% versus 1.2%; $P < 0.0005$), despite the general decrease of *Proteobacteria*. Similarly,

FIG 2 Prevalence of OTUs in freshwater and saltwater across treatments, measured as the proportion of samples where each OTU made up more than 1% of reads. Only bacterial OTUs which were present in more than 10% of all samples are shown.

Coriobacteriaceae increased (1.6% versus 2.2%; $P < 0.0005$), irrespective of the general decrease in *Actinobacteria*.

Figure 2 illustrates the freshwater-to-saltwater shift in prevalence for the most abundant OTUs. Although OTU4 (classified as *Corynebacterium*) showed a major decrease in prevalence from freshwater to saltwater (44% versus 0.61%), this OTU did not show a significant relative quantitative decrease (0.087% versus 0.12%; $P = 0.99$). OTU18 (*Pseudomonas*) decreased in prevalence (65.8% versus 0.6%) as well as relative quantity (1.5% versus 0.0%; $P < 0.0005$). The OTUs with the largest freshwater-to-saltwater increase were OTU13 (*Bradyrhizobium*), with a prevalence of 6% versus 52.4% and a relative quantity of 0.01% versus 1.0% ($P < 0.0005$), and OTU21 (*Lactobacillus*), with a prevalence of 0.0% versus 67.7% and a relative quantity of 0.26% versus 1.2% ($P < 0.0005$). All the OTUs showing major freshwater-to-saltwater shifts also had closely related sequences in the Scottish data set (Table S1).

There was a more even distribution of rarefaction curves for saltwater than for freshwater samples, with more highly abundant OTUs in saltwater (Fig. S1). Water type also showed significant differences in alpha diversity, with saltwater showing higher index levels than freshwater (Fig. 3A and B), while beta diversity showed higher levels in freshwater than in saltwater (Fig. 3C). Using quantitative PCR, we also identified a major (>100-fold) increase in the ratio of bacterial DNA to eukaryotic DNA from freshwater-to-saltwater transition, as determined from small-subunit (SSU) gene copies (Fig. 3D).

Amplicon sequencing of eukaryotic SSU genes from freshwater revealed that >95% of the eukaryotic sequences belong to salmon. By gel electrophoresis we found DNA with a size distribution with bands about 180 bp apart, resembling DNA from apoptotic cells (Fig. S3).

Diets (vegetable oil [VO]- versus marine oil [MA]-based feed) and feed switch did not significantly affect the microbiota composition, either in the freshwater or in the saltwater phase. ANOVA showed no significant main effects for any of the feeding regimens on the overall microbiota composition. Furthermore, diet did not show any effect on alpha diversity (Fig. 3A and B), while there was a slight but significant effect on beta diversity for marine oil in freshwater (Fig. 3C).

Overlap in microbiota across freshwater and saltwater. For the overall overlap in OTUs, we found that 818 OTUs (69%) were shared across freshwater and saltwater.

FIG 3 Alpha diversity, beta diversity, and quantity of the microbiota in freshwater and saltwater. (A and B) Alpha diversity was determined by the Shannon and Simpson indices, respectively. (C) Bray-Curtis was used to determine beta diversity. (D) The quantities of prokaryotes were determined relative to the level of eukaryotic DNA based on SSU gene copies. VO/MA, comparison between vegetable oil and marine oil; VO->MA, switch from vegetable oil to marine oil; MA->VO, switch from marine oil to vegetable oil. ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$.

However, the number of unique OTUs was higher for freshwater than for saltwater, at 245 (21%) versus 117 (10%), respectively. Of the OTUs shared across freshwater and saltwater, a subset of 408 OTUs (34%) were also shared with a Scottish freshwater data set consisting of commercial and aquarium breed parr kept on different feeding regimens (7). Furthermore, 38 (3.2%) of the Scottish OTUs were uniquely shared with the freshwater data set and 14 (1.2%) with saltwater.

Overall, the abundant OTUs (>1% within an individual) were more prevalent in saltwater than in freshwater (Fig. 4). There were four bacterial core OTUs (OTU1, OTU2, OTU6, and OTU10) affiliated with the *Firmicutes* that were abundant in more than 90% of the fishes in both freshwater and saltwater. All the core OTUs showed positive relative quantitative co-occurrence across fishes in both freshwater and saltwater (Fig. 5A and B), in addition to a general increase in relative quantity from freshwater to saltwater (Fig. 5B). All the core OTUs also showed close matches (>97% identity) to OTUs from the Scottish data set (Table S1).

DISCUSSION

We found that the freshwater-to-saltwater transition had a major effect on the microbiota composition, while marine or vegetable oil in the diet had only a minor effect. Salinity represents a major environmental barrier for microbes (15). The freshwater gut microbiota was the least mature, having a lower bacterial load, lower alpha diversity, and sharing of core OTUs, in addition to higher levels of low-abundance OTUs and higher beta diversity than in saltwater. A recent study showed an apparent opposite diversity pattern for the salmon skin microbiota, with higher alpha diversity

FIG 4 Association between respective prevalence of OTUs present at >1% in both saltwater and freshwater. Overlap between core OTUs found in more than 90% samples in freshwater and saltwater is shown.

in freshwater than saltwater (6). For the skin microbiota, the diversity difference is explained by the freshwater microbiota being more mature than the saltwater microbiota (6). A potential explanation for the saltwater maturity difference between skin and gut microbiota could be that the gut microbiota is more protected toward the direct contact with the saltwater than the skin microbiota, which allows continued maturation through the freshwater-to-saltwater transition.

Since LC-PUFA is required in high relatively quantity in freshwater (5), the low-density immature fresh water microbiota would most likely not be sufficient to support the LC-PUFA requirement. We therefore find it unlikely that the gut microbiota plays an important role in alleviating limitations in LC-PUFA in freshwater ecosystems.

We found a dominance of *Firmicutes* at both the parr and postsmolt stages, while wild salmon were dominated by *Proteobacteria* for the corresponding life stages (10). The difference in the ratio of *Firmicutes* to *Proteobacteria* between wild and farmed salmon resembled that with high- and low-fat diets, where a high-fat diet increased the ratio of *Firmicutes* to *Proteobacteria* (16). Thus, the differences in gut microbiota between wild and farmed salmon could partly reflect the high fat and energy content in the farmed salmon feed compared to that of the natural diet (17).

A subset of 4 OTUs showed high stability for the freshwater-to-saltwater transition. Stability across the transition may indicate strong host associations of the core OTUs in the salmon gut, despite the major shift in the overall microbiota. The core genus *Vagococcus* is related to mucin-utilizing species (18). Mucin utilization could potentially explain a close host association for the *Vagococcus*-affiliated core OTU (19), while the positive correlations for the rest of the core OTUs may indicate either cross-feeding, syntrophy, or association with other correlated factors. Specific mechanistic studies, however, are needed to determine the underlying cause for the positive correlations of the core OTUs.

Previous studies identifying core OTUs in the salmon gut of farmed salmon, however, suggest a relatively high number and wide diversity of core OTUs (7, 8). These studies include a relatively low number of fish (<50), not covering the freshwater-to-saltwater transition. This may have led to overestimation of core OTUs. However, although we identified the core OTUs in a Scottish data set, in both freshwater and saltwater and under different feeding regimens, the data sets are still too limited to claim universal distribution.

In conclusion, we have shown a major shift in microbiota composition, diversity, and

FIG 5 Scatterplot matrices for percentages of core OTUs in freshwater (A) and in saltwater (B) and relative quantity (C). Correlations between the relative abundance of core OTUs were determined using Spearman correlations for freshwater (A) and saltwater (B). Differences in levels of OTUs were determined by Kruskal-Wallis test (C). ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$.

quantity for the freshwater-to-saltwater transition, with four core bacteria showing high prevalence and co-occurrence across this transition.

MATERIALS AND METHODS

Fish maintenance and sampling procedure. Fish were sampled from two replicate fish tanks where they were fed vegetable oil (VO)-based or marine oil (MA)-based feed (total of 4 tanks). VO-based feeds contained a combination of linseed oil and palm oil at a ratio of 1.8:1, and MA-based feeds contained only North Atlantic fish oil. A feed switch to the alternative diet was introduced for half of the fish in freshwater (parr stage; approximately 50 g) and then repeated as the fish transitioned into seawater (postsmolt; approximately 200 g). Smoltification was triggered by 5 weeks of winter-like conditions with 12 h of light per day, followed by spring-like conditions with 24 h of light per day. Salmon were then immediately switched to saltwater and allowed to acclimate for 3 weeks before first sampling (5). Gut microbiota sampling was conducted immediately before the feed switch (day 0) in both freshwater and saltwater and at days 1, 2, 6, 9, 16, and 20 after the switches. The experimental setup is schematically outlined in Fig. 6.

Sampling and DNA extraction. The sampling procedure involved antiseptically squeezing out the complete gut contents by using tweezers. Gut content samples were collected in 2-ml sample tubes (Sarstedt, Germany) prefilled with ~0.2 g of acid-washed beads ($\leq 106 \mu\text{m}$ in diameter; Sigma-Aldrich,

FIG 6 Outline of the experimental setup. For each experimental period the fishes were given a diet based on either vegetable or marine oil. The numbers of samples (*n*) analyzed for each feeding category are included.

Germany) and 400 μ l of stool transport and recovery buffer (Roche, Germany) before long-term storage at -40°C .

Samples ($n = 180$ from freshwater and $n = 169$ from saltwater) were thawed and homogenized by bead beating in a MagNA Lyser instrument (Roche, Germany) for 2×20 s at 6,500 rpm with a 1-min rest between runs. DNA was isolated using an LGC Mag Midi DNA extraction kit (LGC Genomics, UK) according to the manufacturer's instructions. Extracted DNA was quantified with a Qubit double-stranded DNA (dsDNA) HS assay kit (Thermo Fisher Scientific, USA) and analyzed on a 1% agarose gel.

Quantitative PCR. To determine the number of eukaryotic and prokaryotic SSU genes, quantitative PCR was performed using LightCycler 480 II (Roche, Germany), with primer pairs PRK341F (5'-CCTACG GBRBGCASCAG-3')/PRK806R (5'-GGACTACYVGGGTATCTAAT-3') (20), targeting the V3-V4 region of the prokaryotic SSU gene, and 3NDF (5'-GGCAAGTCTGGTGCCAG-3') (21)/V4EukR2 (5'-ACGGTATCTATCCTTCG-3') (22), targeting the V4 region of the eukaryotic SSU gene. Reactions were performed in 20- μ l volumes containing 1 $\times$ Hot FirePol EvaGreen qPCR Supermix (Solis BioDyne, Estonia), 0.2 μ M each primer, and 1 μ l (0.2 to 30 ng) of genomic DNA. Thermal conditions involved initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C (in PCR targeting prokaryotes) or 59°C (in PCR targeting eukaryotes) for 30 s, and elongation at 72°C for 45 s.

Illumina sequencing. The taxonomic composition of the microbiota was determined by sequencing the resulting amplicons from a two-step PCR using the same primers as used in quantitative PCR. Amplification was performed in 25- μ l volumes containing 1 $\times$ HotFirePol blend master mix (ready to load; Solis BioDyne), 0.2 μ M concentrations of both primers (Thermo Fisher Scientific, USA), and 2 μ l (0.4 to 60 ng) of genomic DNA. First PCR was performed with initial denaturation at 95°C for 15 min, followed by 30 cycles of identical denaturation, annealing, and elongation steps as done in qPCRs. A final elongation at 72°C for 7 min was included. The resulting amplicons were purified with AMPure XP beads (Beckman-Coulter, USA) by following the manufacturer's instructions. For attachment of dual indices and Illumina sequencing adapters, a second PCR was performed with Illumina-modified prokaryote and eukaryote primers under the same conditions as before, only with 12 cycles and an increased annealing step to 1 min. Amplicon libraries were quantified by Qubit dsDNA HS assay kit and normalized to a sequencing pool before purification by AMPure XP beads. The final library was quantified in a QX200 droplet digital PCR system (Bio-Rad, USA) using primers targeting Illumina adapters, following the manufacturer's recommendations. Sequencing was performed on a MiSeq platform (Illumina, USA) using v3 chemistry with 300-bp paired-end reads.

The resulting amplicon reads were processed (demultiplexing, primer removal, merging, filtering, dereplicating, OTU clustering, and filtering of chimeras) using a standard procedure associated with USEARCH 9.0 software (23), with taxonomic assignments using the RDP database (24) and BLAST for eukaryotic SSU genes (25). Comparison between these data and an additional Scottish prokaryotic SSU data set (7) was done using BLAST with representative sequences for the OTUs toward a database for the Scottish SSU sequences. A match was assigned if the hit length was >300 bp and identity was $>97\%$. Read counts and characteristic sequences for OTUs are available at http://www.fairdomhub.org/data_files/1585.

Data analysis. OTU data were analyzed in the R computing environment (<https://www.r-project.org/>). For each sample we computed the taxonomic profile as follows. For sample i ($i = 1, \dots, N$) and OTU j ($j = 1, \dots, P$), we have the read-count c_{ij} . For each sample, we compute the relative abundance as follows:

$$r_{ij} = \frac{c_{ij} + q}{\sum_{j=1}^P (c_{ij} + q)}$$

where q is a pseudocount added to all read counts, required below. We used a q value of 1 in this analysis. The vector of relative abundances for a sample is an example of compositional data, and for such data a commonly used transform is the Aitchison log-ratio transform (17):

$$x_{ij} = \log_2 \left(\frac{r_{ij}}{\left(\prod_{j=1}^P r_{ij} \right)^{1/P}} \right)$$

Thus, the taxonomic profile value x_{ij} is the logarithm of the relative abundance divided by its geometric mean. The pseudocounts added are essential to avoid zeros in the denominator of this transform. This transform is often beneficial when later using some kind of sum-of-squares analysis (e.g., principal-component analysis [PCA], ANOVA, or Euclidean distances) (17). For sample, i in the vector $x_i = (x_{i1}, \dots, x_{ip})$ was arranged as row number i in the OUT matrix X of taxonomic profiles (N rows and P columns).

Based on the matrix X , we used PCA to get an overview of the variations in taxonomic profiles. More specifically, the PCA scores of the first components were used in ANOVA to test for effects of water type W (fresh, salt), diet D (vegetable oil, vegetable-to-fish oil, fish oil, fish oil to vegetable oil), and sampling day S (0, 1, 2, 6, 9, 16, and 20):

$$y_{ijkl} = \mu + W_i + D_j + S_k + e_{ijkl}$$

where $i = 1, 2, j = 1, \dots, 4$, and $k = 1, \dots, 7$. As the response y_{ijkl} , we used PCA scores from components 1, 2, ..., 5 in turn, reflecting different aspects of change in microbiota composition.

We used the Kruskal-Wallis test for nonparametric comparison of means. False-discovery rate (FDR) correction was done using the Benjamin and Hochberg approach (26).

Accession number(s). The raw data reads obtained from the 16S rRNA gene sequencing are available in the Sequence Read Archive (SRA) database under accession number SRP119730 (<https://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP119730>).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01974-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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