Insights into the circulating microbiome of the Atlantic and Greenland halibut populations: the role of species-specific and environmental factors

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Article

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Abstract

The establishment of long-term microbiome-based monitoring programs is critical for the management and conservation of wild fish populations in response to climate change. In most cases, these studies have been conducted on gut and, to a lesser extent, skin (mucus) microbiomes. Here, we exploited the concept of liquid biopsy to study the circulating bacterial microbiome of two Northern halibut species of economic and ecological importance. Amplification and sequencing of the 16S rRNA gene was achieved using a single drop of blood fixed on FTA™ cards to identify the core blood microbiome of Atlantic and Greenland halibut populations inhabiting the Gulf of St. Lawrence, Canada. We provide evidence that the circulating microbiome DNA (cmDNA) is driven by both species-specific and environmental factors. More specifically, we found that the circulating microbiome signatures are species specific and vary according to sex, size, temperature, condition factor, and geographical localization. Overall, our study provides a novel approach for the detection of dysbiotic signatures and the risk of disease in wild fish populations for fisheries management, most notably in the context of climate change.

Introduction

The Atlantic halibut *Hippoglossus hippoglossus* (Linnaeus, 1758) *(H. hippoglossus)* and the Greenland halibut *Reinhardtius hippoglossoides* (Walbaum, 1792) *(R. hippoglossoides)* are two species of flatfish widely distributed in the Northwest Atlantic and characterized by distinct populations in the Gulf of St. Lawrence (GSL) [1,2]. These populations support the two most valuable groundfish fisheries in the region and are biannually assessed to provide scientific advice for management [3,4]. The Atlantic halibut abundance has been steadily increasing, whereas the Greenland halibut stock has declined over the last two decades. Although the reasons for these fluctuations are not fully elucidated, the rapid warming of the deep channels of the GSL [3,5] and increased competition by redfish for Greenland halibut [6,7] are considered the primary factors driving these changes. The development of sensitive and predictive biomarkers is essential for a close follow-up of the health status of these stocks.

The microbiome, or the pool of nucleic acids from microbes that can be found in a host species, has attracted considerable attention from scientists in recent years as a predictive biomarker to assess the health status of an individual [8]. The microbiome is an important determinant of the health status of an organism, as it contributes to the regulation of several physiological processes, such as the immune response and host energy metabolism.

Although most studies on the use of microbiome signatures as a predictive tool were initially performed in clinical settings, the democratization of next-generation sequencing (NGS) technologies for the analysis of 16S ribosomal RNA (rRNA) gene amplicons has facilitated its application in different research fields, including studies in fish populations [9]. Studies on the skin and gut bacterial microbiomes of fish have shown that a balanced microbiome plays a critical role in the host's health, providing protection against pathogens while bringing nutritional benefits [10]. Disruption of this balance, which is often

referred to as dysbiosis, changes the biodiversity and abundance of specific bacterial communities, often leading to health complications [11].

Considering their economic and ecological importance, an increasing number of studies have thus focused on defining the microbiome signatures of Teleost. These studies have shown that this signature is dependent on several factors, including host genetics, morphometrics, and a number of environmental factors, including biotic and abiotic factors [12,13]. In most cases, however, these studies have been conducted in laboratory/experimental settings or in fish farms. Studies in wild fish populations remain relatively scarce. Moreover, most of these studies have been performed on the gut microbiome and, to a lesser extent, skin (mucus) microbiomes [13–15]. In recent years, however, the concept of a circulating microbiome has emerged as an interesting alternative to invasive, lethal, and logistically challenging tissue biopsies. Even if blood has historically been considered exempt from microbes in healthy individuals for years, it is now irrefutable that bacterial, viral, fungal and other microorganism genomes are present in the blood (blood-cell or plasma) [16–20]. This feature thus allows us to study the microbiota of an organism without the need for tissue biopsy. The concept of the circulating microbiome is thus particularly well adapted to the development of routine and predictive biomarkers. The utility of this approach has recently been demonstrated in clinical settings, offering a new perspective for the development of biomarkers in ecology [21–24] similar to those described for several diseases in humans [16,20,25]. In fact, the existence of a blood microbiome is a concept that is now widely accepted not only in humans but also in animals, including pigs, broiler chickens, camels, cows, goats, cats and dogs [21,23,24,26–29].

In the present work, we have characterized, for the first time, the blood 16S rRNA microbiome signatures of two wild fish populations of ecological and economical interest from the GSL, the Atlantic halibut *H. hippoglossus* and the Greenland halibut *R. hippoglos*, and examined physiological and environmental factors that impact their microbiome signature.

Material & Methods

Sampling

Blood samples from Greenland halibut (n = 97; 316.16 ± 15.08 mm) and Atlantic halibut (n = 86; 762.04 ± 30.13 mm) were collected between August 15th and October 1st 2019 during the annual DFO bottom trawl surveys performed in the northern and southern sectors of the GSL, Canada (**Table 1**). Scanmar® hydroacoustic sensors attached to the trawl and a conductivity, temperature, and depth (CTD) probe were used to record temperature. Blood samples were taken immediately upon trawl retrieval, and liquid biopsies were performed at 56 sites for at least one species (Fig. 1). The number of liquid biopsies performed per station (ranging from 1 to 14) was opportunistic and depended on the presence of halibut and workload at a given site. Blood samples were collected with a heparin-coated 3-mL sterile syringe and a 22-G needle following a dorsal incision using a sterilized knife. Drops of blood were collected and

immediately stored on a Flinders Technology Associates (FTA™) card (SigmaAldrich, Oakville, ON, Canada). Samples were allowed to air dry and kept afterward in a plastic bag with a desiccant, as described in Caza *et al*. (2019). The sex of each sampled individual was determined by visual identification of the gonads following the dissection of specimens by the DFO science crew. The care and use of field-sampled animals complied with the Government of Canada animal welfare laws, guidelines and policies as approved by Fisheries and Oceans Canada. All methods are reported in accordance with ARRIVE guidelines

DNA extraction, amplification and sequencing

All procedures involving DNA extraction and purification were conducted in a white room where pressure, temperature, and humidity were controlled to minimize contamination. Individual discs were cut from the FTA™ cards using a sterile 5.0-mm single round hole punch, and total DNA was isolated using the QIAamp DNA Investigator Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol. Quantification of DNA was performed in duplicate using a Quant-iT™ PicoGreen® dsDNA detection kit (Molecular Probes, Eugene OR, USA). Amplification of the V3-V4 region of the 16S ribosomal RNA (rRNA) and 16S gene amplicon sequencing for all DNA samples were performed at Centre d'Expertise et de Services Génome Québec (Montréal, QC, Canada) using the universal primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). Sequence libraries were prepared by Genome Quebec with the TruSeq® DNA Library Prep Kit (Illumina, San Diego, CA, USA) and quantified using the Kapa Library Quantification Kit for Illumina platforms (Kapa Biosystems). Paired-end sequences were generated on a MiSeq platform PE300 (Illumina Corporation, San Diego, CA, USA) with the MiSeq Reagent Kit v3 600 cycles (Illumina, San Diego, CA, USA). Raw data files are publicly available on the NCBI Sequence Read Archive (PRJNA853332).

16S rRNA data processing

Illumina sequence data (FASTQ files) were trimmed using *Cutadapt* (version 2.8). The 16S rRNA (V3-V4) amplicon sequence variants (ASVs) were generated with the DADA2 pipeline (version 1.16.0; Callahan *et al.* (2016)) and subsequently within the R environment (R version 4.0.3, Team (2021)). The RDP 16 database was used for ASV assignment. The software packages *phyloseq* (1.36.0), *microbiomeSeq* (0.1), *microbiomeMarker* (0.99.0) and *vegan* (2.5.7) were used to characterize the microbial communities [30–33]. The maps were created with the packages *ggplot2* (3.3.6) and *rnaturalearth* (0.1.0) [34]. An ASV was considered part of the core microbiome if it had a minimum prevalence (rate of presence in the group of samples) of 70% with a detection threshold of 0.01% of relative abundance, as noted in Palanisamy et al. (2022). A similar decision tree was applied for the core genus (abundance of ASVs of the same genus were summed) but with 90% prevalence. We chose not to limit the core microbiome at 100% prevalence because the study was not under controlled conditions; rather, it was performed on a wild population where greater variations are expected, especially with this sample size. The prevalence varies between studies of the core microbiome, ranging from > 10–100% [22,35–40]. A stringent threshold at 90% was chosen as reported in previous studies [39–41].

Classes based on environmental and morphometric data

Individual fish were classified into categories to assess environmental and ontogenetic effects and were assigned as mature or immature according to known length at which 50% of males had reached reproductive size (L50), i.e., 360 mm for Greenland halibut [3, 7] and 920 mm for Atlantic halibut [42]. Given that sex information was missing from many individuals, the male L50 was chosen, as it is lower, so the immature individuals would have less chance to be mislabeled as mature. Size classes, based on the diet of the fish, were defined as previously described [43]. Briefly, four classes were defined for the Greenland halibut: (1) Class 1, small prey for individuals smaller than 200 mm; (2) Class 2a, intermediate prey 1 for individuals between 200 and 300 mm; (3) Class 2b, intermediate prey 2 for individuals between 300 and 400 mm and (4) Class 3, large prey for individuals larger than 400 mm. Classes 2a and 2b were merged because given the low total number of fish. It was not possible to define length classes for the Atlantic halibut, as the number of individuals consuming smaller prey was too low. Classes were also defined according to the water temperature. Temperatures below than 5°C were considered “cold water”, and temperatures above 5°C were considered “warm water” [7]. Given that the Atlantic halibuts’ range of temperature tolerance was wider than the temperature measured, they were not included in this analysis. Finally, the relative condition K factor was also calculated based on the length and weight of each individual [44,45]. A linear regression was performed between $\log_{10}$ (weight) and $\log_{10}$ (length) as follows:

$$log_{10}\left(W\right) = log_{10}\left(a\right) + b * log_{10}\left(L\right)$$

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where W is the weight, L is the length and a and b are constant coefficients.

The coefficients a and b were calculated and used to estimate an expected weight W_e of each individual based on their length with the following equation:

$$W_e = aL^b$$

Finally, the K_{rel} of Le Cren was calculated as follows:

$$K_{rel} = \frac{W}{W_e}$$

The autocorrelation, the homoscedasticity of the error terms and the normal distribution of the residuals of the linear regression were validated with statistical analysis (Durbin Watson, Breusch–Pagan and Shapiro–Wilk tests). Classes were created with $K_{rel}>1$ corresponding to the “high condition” and $K_{rel}<1$ corresponding to the “low condition”.

Spatial analysis

Overall, five geographical zones were defined. Spatial zones were created to separate different habitats in the Gulf based on sample sites with similar characteristics, e.g., depth, temperature, and spatial closeness. The Estuary-Western Gulf area extends from 65°W to 69°W longitude and from 49.7°N to 51°N latitude, and the Northeast Gulf area is situated between 57°W and 63.7°W longitude and 49°N and 51°N latitude. The Lawrence channel area is between longitudes 59°W and 65°W and latitudes 47°N and 49.7°N with sample sites deeper than 110 m (included). The Chaleur Bay area includes sampling sites between 64°W and 67°W longitude and 47.5°N and 48.5°N latitude. Finally, the southern Gulf area is located between 60.5°W and 65.5°W longitude and 45.5°N and 48°N latitude with sampling sites less deep than 110 m (not included). In the Greenland halibut, the number of fish per area was n = 22 for the Estuary-Western Gulf t, n = 19 for the Chaleur Bay, n = 22 for the Northeast Gulf and n = 34 in the Laurentian Channel. Regarding Atlantic halibut, the number of fish was n = 14 for the Estuary-Western Gulf, n = 11 for the Southern Gulf, n = 17 for the Northeast Gulf and n = 44 for the Laurentian Channel.

Statistical analysis

Bacterial taxonomic α-diversity (intrasample) was estimated using the richness and the Shannon and Simpson indices as implemented in the R package *microbiome* (1.14.0). Variation in bacterial α-diversity and taxa abundances between the two species were assessed using either the Kruskal–Wallis test or Wilcoxon-Mann–Whitney test given that none of the variables exhibited a normal distribution. In addition, α-diversity was also calculated among classes according to the length, temperature and condition factor K [45]. The Kruskal–Wallis test was followed by a pairwise Wilcoxon-Mann–Whitney test if the p value (p) was significant ($p < 0.05$). The β-diversity (intersample) was estimated using phylogenetic weighted UNIFRAC dissimilarities assessed by principal coordinates analysis (PCoA). Differences in community composition were tested using permutational multivariate analysis of variance (PERMANOVA) for weighted UNIFRAC indices with 999 permutations, as implemented in the R *vegan* package (2.5.7) or the *pairwise Adonis* package (0.4). Detailed statistical analyses on variations with morphometric and environmental data are presented as Supplementary Information. Differences were considered statistically significant at $p < 0.05$. Linear discriminant analysis (LDA) effect size (LEfSe) was performed on the microbiome of each species and on the different classes to highlight discriminative taxa for each class. This analysis was performed using the *microbiomeMarker* package (0.99.0). The cutoff was chosen at an LDA score of $\log_{10}$ (LDA score) ≥ 4. All analyses were performed in R studio (v4.0.5).

Results

Preliminary characterization of the cmDNA

A total of 183 blood samples of Atlantic halibut and Greenland halibut were collected at the end of summer and early fall of 2019 in the Gulf of St. Lawrence (Fig. 1). The cmDNA signatures were determined by sequencing the V3-V4 hypervariable regions of the 16S rRNA gene. Approximately 6 million raw reads were retrieved after filtering (2.5 and 3.5 million for Atlantic halibut and Greenland halibut, respectively). The number of sequences per sample ranged between 3 985 and 55 077. The mean

numbers of reads per individual were 35 575 ± 971 and 29 296 ± 1 185 for Greenland halibut and Atlantic halibut, respectively. The number of ASVs per sample curve confirmed that the depth of sequencing was sufficient to plateau the number of ASVs (**Figure S1**). A total of 7 105 unique ASVs were obtained, including 7 102 that were identified as of bacterial origin (3 of archaeal origin were removed from the analysis). A total of 6 450 ASVs were greater than or equal to 0.01% relative abundance. Overall, 3362 ASVs were present in Atlantic halibut (112 ± 7 per individual) and 5 023 in Greenland halibut (with an average of 161 ± 9 per individual).

Differences in the circulating microbiome at the phylum level

Overall, 30 different phyla were identified (23 and 29 in Atlantic halibut and Greenland halibut, respectively), 63 classes (44 and 59), 121 orders (88 and 112), 241 families (189 and 224) and 685 genera (473 and 587) (**Table II**). At the phylum level, the blood microbiome signature was dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Fig. 2). The mean relative abundance of *Proteobacteria* was however significantly higher in Greenland halibut (81.6 ± 1.5% versus 62.4 ± 2.3% in Atlantic halibut) (Wilcoxon-Mann–Whitney test; $p < 0.001$). Accordingly, the other three main phyla were significantly higher in Atlantic halibut at 9.3 ± 0.9% versus 6.9 ± 0.8% for *Firmicutes* ($p = 0.032$), 6.6 ± 0.7% versus 4.4 ± 0.5% for *Bacteroidetes* ($p = 0.044$) and 4.0% ± 0.5% versus 2.6 ± 0.4% for *Actinobacteria* ($p = 0.0028$) (Fig. 2A). The mean relative abundance of uncharacterized bacteria was nonetheless important in Atlantic halibut, accounting for ~ 15% on average. This finding is even more obvious based on the individual relative abundance with some individuals possessing 60% of uncharacterized bacteria (Fig. 2B**)**. The microbiome structures with other taxa are provided in Supplementary Figures S2-S3.

The genus-level core circulating microbiome

Three genera, *Pseudoalteromonas, Psychrobacter* and *Acinetobacter*, were present in 90% of samples for both species with an aggregation of the genera. In Atlantic halibut, these genera were found to be the most abundant, accounting for 12.9%, 12.1% and 8.7% of the mean relative abundance, respectively. A fourth genus that was present in 90% of samples was *Staphylococcus* with a mean relative abundance of 3.2% (Fig. 3A). In Greenland halibut, these three genera represented 23.6%, 16.6% and 3.4% on all species average, respectively, whereas *Vibrio* was the fourth core genus with 6.9% mean relative abundance. In total, 685 genera were found, 375 of which were present in both halibut species (54.7%) (Fig. 3B). Although the majority of the genera found at 50% prevalence were shared by both species, some genera were unique in each species, such as *Enhydrobacter* in Atlantic halibut and *Oleispira* in Greenland halibut (Fig. 3C). This distribution of genera differed between the two species; major differences were the higher abundance of *Vibrio* and the lower abundance of *Acinetobacter* in Greenland halibut. The number of genera present with a prevalence of 50% was also higher in Greenland halibut than Atlantic halibut.

Comparative analysis of the bacterial diversity between species

We next compared the overall biodiversity of the cmDNA between both species using a UniFrac-based PCoA that showed that the microbiomes tended to cluster according to species, but with high variations within species composing the microbiome and a low percentage of variance explained by the two axes (32.8%) (Fig. 4A). PERMANOVA confirmed the significant difference between the two halibut species ($p < 0.001$), but the effect size was very low for the groups ($R^2 = 0.06$) compared to the residuals ($R^2 = 0.94$), suggesting that it was not a strong determinant between these fish microbiomes. These findings support a recent study showing that the gut microbiome of flounders (*Pleuronectidae)* is similar among family members (Huang et al., 2020). Regarding α-diversity indicators, no significant differences in evenness or diversity were noted between the two species; the mean Simpson index (Atlantic halibut, 0.11; Greenland halibut, 0.13) and the mean Shannon diversity index (Atlantic halibut 3.36; Greenland halibut, 3.37) were equivalent (Fig. 4B). The richness index however was higher in the case of Greenland halibut with an average of 161 ± 9 ASVs per individual compared to 112 ± 7 ASVs per individual for Atlantic halibut ($p < 0.001$).

LEfSe comparison of the two species

To further investigate the differences between the circulating microbiomes of the two halibut species, an LEfSe analysis was performed with a cutoff at $\log_{10}$ (LDA score) ≥ 4. This criterion allowed us to observe several differences in the structure of the two blood microbiomes at the phylum and genus levels (Fig. 4C **& D**). In particular, *Proteobacteria* were found in higher abundance in Greenland halibut, whereas *Actinobacteria*, *Firmicutes* and *Bacteroidetes* were more abundant in Atlantic halibut (Fig. 4C). At the genus level, more genera were discriminative for Greenland halibut (Fig. 4D). These genera included the core genera *Pseudoalteromonas* and *Psychrobacter* or *Vibrio* and other genera, such as *Photobacterium*, *Burkholderia* and *Escherichia/Shingella.* The core genera *Staphylococcus* and *Acinetobacter* were discriminative exclusively for Atlantic halibut.

Correlations with biotic and abiotic factors

We examined whether the blood microbiome signature within each fish species varied according to different variables, including maturity classes (length), water temperature (measured at the sampling depth of the trawl), sex, and Fulton's condition factor (K). We found that the blood microbiome structure varied depending on the maturity stage for both species at the phylum and genus levels. In the case of Greenland halibut, significant differences in the abundance of *Corynebacterium*, *Staphylococcus*, *Burkholderia*, *Pseudoalteromonas*, *Psychrobacter* and *Vibrio* were noted (**Figure 5A, Figure S4**). The most important difference between the mature and immature classes was in the case of *Vibrio*, which decreased by almost 3-fold in the mature class (from 9.7–3.3%; $p < 0.001$) (**Table III)**. The difference in *Burkholderia* between mature (0.7%) and immature (4.5%) Greenland halibut was also significant ($p < 0.001$). In the case of Atlantic halibut, the only two genera that were discriminative between both classes

were *Psychrobacter* and *Escherichia/Shingella* (Fig. 5B, **Figure S5)**. We also found significant differences in the diversity of the blood microbiome between mature and immature classes. In the case of Greenland halibut, we found a significant difference in the richness index (Fig. 5C). Significant differences in the β diversity between mature and immature plants were also found for both halibut species (**Figure S6 and S7**).

We next assessed whether temperature had an impact on the circulating microbiome signatures. Given the limited temperature tolerance of Greenland halibut [47], we divided the populations into two groups based on whether they were sampled at temperatures below or above 5°C. Our results showed significant temperature-related differences in Greenland halibut at both the phylum and genus levels (Fig. 6A, **Figure S6**). The relative abundance of *Pseudoalteromonas* was 34.4% in specimens distributed in relatively cold waters, compared to 19.9% in those in relatively warm water ($p = 0.005$) (**Table IV**). The relative abundance of *Vibrio* was also lower in Greenland halibut distributed in warmer water (9.8%) than in cold water (5.9%) ($p = 0.001$). Similar findings were observed in the case of *Pseudoalteromonas* (**Figure S8**). Again, we found significant variations in the α and β diversity between temperature classes for Greenland halibut (**Figures S6 and S9**).

We next determined whether the circulating microbiome varied in association with the size class of the Greenland halibut. Discriminative taxa included *Psychrobacter*, which was more abundant in fish eating small prey (Fig. 6B), with the highest mean relative abundance of 27.9% in this group (Kruskal—Wallis; $p < 0.001$) (**Table V**). The two other core genera were identified as discriminative in intermediate-sized prey-eating fish with a mean relative abundance of 10.4% for *Vibrio* ($p < 0.001$) and 37.2% for *Pseudoalteromonas* ($p < 0.001$). *Corynebacterium*, *Staphylococcus*, *Acinetobacter* and *Burkholderia* were discriminative for large prey-eating fish (**Figure S10**). All these genera were previously documented in the gut or skin microbiome of teleost fishes, and *Acinetobacter* was identified as a biomarker of carnivorous fish (Huang et al. 2020).

With regard to differences between males and females, we found only a few statistically significant differences (**Figure S11**). *Shewanella* and *Psychrobacter* were more abundant in Greenland and Atlantic male halibut, respectively, whereas the relative abundance of *Acinetobacter* was more abundant in Atlantic halibut females. This abundance of *Shewanella* in female versus male Greenland halibut was quite significant (0.4% in females compared to 3.2% in males). We did not observe any variations in the α and β diversity between males and females for either species (**Figures S6, S7, and S9**).

Finally, according to the factor K conditions, only *Photobacterium* was highlighted as a discriminative genus for low-condition Atlantic halibut, whereas *Streptococcus* was a marker of high-condition individuals (**Figure S12**). In Greenland halibut, the only significant difference was the lower abundance of *Escherichia/Shingella* in the Greenland halibut with a low relative condition factor (K).

Spatial variation

We next assessed whether variations in the circulating microbiome could be attributed to geographic distribution. LEfSe analyses showed several variations at the genus level in both halibut species (Fig. 7A, B). More specifically, in the case of Greenland halibut, *Photobacterium* and *Burkholderia* were significantly more abundant in the Laurentian Channel than in the other areas with mean relative abundances of 13.2 ± 2.8% ($p < 0.001$) and 5.2 ± 1.0% ($p < 0.001$), respectively (Fig. 7C). *Exiguobacterium* and *Oleispira* were also more abundant in the Northeast Gulf ($p < 0.001$), whereas *Oleispira* was especially abundant in the Estuary and Western Gulf ($p < 0.001$). This finding contrasted with that noted *Psychrobacter*, which was equally also abundant in those both areas. Among other notable differences in Greenland halibut, we found a higher abundance of *Vibrio* in the Chaleur Bay and Northeast Gulf ($p < 0.001$). In the case of the Atlantic halibut, the Southern Gulf was characterized by a higher abundance of *Bizionia* ($p = 0.009$) and *Neorickettsia* ($p = 0.015$) relative to that in the Northeast Gulf and a lower abundance of *Staphylococcus* compared to the Estuary and Western Gulf ($p = 0.039$). In terms of biodiversity, we did not find notable differences among areas with the exception of a lower richness in the Laurentian Channel compared to Chaleur Bay (**Figure S9**).

Discussion

The circulating microbiome is an emerging concept that has drawn a high level of interest in the biomedical field given its potential to generate predictive biomarkers and the means for the detection of pathogens. In the present work, we applied this concept to characterize the circulating microbiome signature to two wild halibut populations of economic and ecological importance. We further studied how the microbiome signature composition and structure correlate with physiological and environmental factors.

Typically, in humans and other species, including fish, most microbiome studies have focused on the gut microbiome. In addition to ethical and logistical considerations, recent studies in humans and other species have unequivocally shown that defining the core blood microbiome using a single drop of blood exhibits considerable potential as a disease biomarker [16,20]. The core blood microbiome can also be used to detect potential pathogens given that pathogenic (and symbiotic) bacteria are not exclusively found in the gut but also in other tissues. It is important to note, however, that the presence of bacteria in the blood is not *de facto* associated with a disease state, as the existence of a healthy blood microbiome is increasingly recognized [16]. Indeed, the paradigm that the blood is a sterile compartment has shifted radically since the development of 16S rRNA next-generation sequencing methods. However, it is not clear at present whether the blood microbiome reflects bacteria that inhabit the blood in dormancy [16] or bacteria that translocate from one niche to another via the blood circulation, a process referred to as “atopobiosis” [48]. According to this hypothesis, not only do bacteria use blood vessels to migrate from one tissue to another, but they can also do so by protecting themselves by infecting erythrocytes or white blood cells. Notwithstanding these fundamental questions, defining a dysbiotic blood microbiome has become a promising avenue for the development of clinical biomarkers [49,50]. Combined with a logistically simple method based on a single drop of blood that can be stored at room temperature on cellulose

paper, our study thus opens the door to the development of a new type of biomarker that could be easily integrated into long-term monitoring programs, most notably in the context of climate change.

From a fundamental point of view, our study has revealed that the blood microbiome of Greenland halibut and Atlantic halibut is unexpectedly richer than that reported to date in the blood of other endotherm animals [21–24], including other fish and marine species [36,37,41,51–59]. In addition, we found close similarities with other core blood microbiomes, as well as skin and gut microbiomes in terms of phyla (*Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*) and genera [12,37,60]. However, a first glimpse at the blood microbiome of these two species revealed that their core blood microbiomes share many features, most notably at the phylum and genus levels. Overall, a total of 30 different phyla, 63 classes, and 685 genera were identified within the cmDNA of both species. At the phylum level, the microbiome signatures of the cmDNA were dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, although both species showed significantly different relative abundances in their phyla. At the genus level, the aggregated core genera were dominated by *Pseudoalteromonas*, *Psychrobacter* and *Acinetobacter* for both fish. The core ASV analysis provides more information on the core bacteria found with the aggregated genera. While the core aggregated genera were based on hundreds of ASVs, each one differed from another, approximately 30% (on average) of the microbiome was represented by only five ASVs for Atlantic halibut and nine for Greenland halibut out of 3362 and 5023 ASVs, respectively (**Figure S13**). As an example, *Pseudoalteromonas* represented an average 23.6% of the Greenland halibut circulating microbiome when taking into account all 132 ASVs characterized as *Pseudoalteromonas*. However, the mean relative abundance is unequal among those ASVs given that one of them represents 18.2% on average and the other only 5.4%. Moreover, no *Vibrio or staphyloccocus* ASV stood out even though the prevalence was lower. This finding indicates that *Vibrio* and *Staphylococcus* vary considerably more among individuals than *Pseudoalteromonas*, *Psychrobacter* and *Acinetobacter*.

Another notable difference between fish circulating microbiomes was the α- and β-diversity, and the richness per individual was higher in Greenland halibut. This microbiome was also more diverse in terms of the number of ASVs (5023 ASVs against 3362 ASVs in Atlantic halibut). We also found distinct genera in each species (*Enhydrobacter* for the Atlantic halibut and *Oleispira*, *Burkholderia* or *Shewanella* among others for the Greenland halibut). All these differences are likely to reflect genetic factors and coevolution history, as noted in the case of the gut microbiome [12,13,37]. Some genera were found before in the fish microbiome. For example, *Enhydrobacter* was identified in the mucus microbiome of three freshwater species [58] and present in at least 50% of the circulating microbiome of Atlantic halibut in the current study. *Shewanella* is another example that was found in high abundance in the gut microbiome of Atlantic mackerel [12] and previously described as an indicator of piscivorous behavior [37]. The environment is another factor that plays a role in the structure of the circulating microbiome. Greenland halibut is a cold-water (1–4°C) species, whereas Atlantic Halibut is characterized by a higher temperature tolerance (1–13°C). Our study also revealed that the relative abundance of the core genera and phyla varied according to size and temperature for Greenland halibut. *Vibrio* and *Pseudoalteromonas* were identified as discriminative taxa for each environmental factor studied. *Vibrio* and *Pseudoalteromonas* were

abundant in cold water and in immature individuals, particularly for fish eating intermediate-sized prey. Additionally, *Psychrobacter* was significantly more abundant in immature fish for both species, especially in Greenland halibut eating small prey and male Atlantic halibut. Finally, *Acinetobacter* varied according to sex in Atlantic halibut and with the size class in Greenland halibut. It is however important to consider confounding factors before drawing conclusions. For example, in our case, the sexual dimorphism implies that males are smaller than females at the same age in both species. Overall, our results indicate that the core blood microbiome is impacted by biotic and abiotic factors, similar to the fish gut microbiome [12,13,60].

An interesting finding was the difference in the core microbiota between males and females. Their microbiome did not differ in α- or β-diversity. However, LEfSe analysis pointed to a genus that discriminated between males and females in Greenland halibut. Specifically, *Shewanella* was more prevalent in male Greenland halibut, as confirmed by the change in relative abundance. This genus did not appear in any other LEfSe for other classes. In humans and animals, sex is among the most important factors that shape the gut microbiome [61]. In fish, such differences between males and females have been reported for the gut microbiome. Indeed, a study in three-spined stickleback and Eurasian perch showed that the diet-associated microbiota is sex dependent [62].

Our LEfSe analysis comparing the blood microbiome between condition classes showed that fish characterized by high condition levels presented significantly more *Streptococcus* than low-condition individuals in Atlantic halibut. In contrast, *Photobacterium* and *Vibrionaceae* were highlighted as discriminative taxa for low-condition Atlantic halibut. Although both genera have been identified in symbionts of other flatfish gut microbiomes [63,64], they also comprise well-known pathogenic species [65,66]. One must take into account however that Fulton's K varies with multiple factors, including age, sex, and seasons.

Finally, our results highlighted spatial differences in the circulating microbiome signatures, which were particularly evident at the genus level **(Figures S14 and S15**). Several factors could explain such variations, including the location of nurseries for Greenland halibuts in some of these areas [67]. The genera correlated with the nurseries area corresponded to those correlated with the immature individuals, i.e., *Psychrobacter* and *Vibrio*. However, it is important to take into consideration potential confounding effects that may play a role in these variations, most notably for *Burkholderia*, which was also found in the large fish that are more common in the Laurentian channel. Although the reasons behind these variations remain unclear, our data provide the basis for further investigation into the role of specific biotic and abiotic factors and how changes in these factors impact the circulating microbiome of these species.

In conclusion, our study provides a reference for future studies aimed at examining the impact of climate change on wild fish populations. Based on the use of a single drop of blood fixed on cellulose paper, the logistically friendly method we used is particularly well adapted for long-term monitoring of fish populations, most notably in response to climate change and the increase ocean temperatures. Moreover,

given the stability of blood DNA on cellulose papers, this approach is perfectly adapted for biobanking purposes, facilitating future spatiotemporal retrospective studies. This study brings a first glimpse of what a circulating microbiome in fish look like, but there are a lot of questions yet unanswered. The similarities with the gut microbiome were striking and should be investigated. Furthermore, sex, maturity, diet and health indicators influenced the microbiome, especially the core microbiome. However, studies in a more controlled environment with finer assessments of maturity, health and diet are needed to confirm these observations. Such studies would also reveal the impact of the environment on the relationship between physiological factors and the circulating microbiome. Future studies at a finer taxonomic level combined with multiomics analysis, including transcriptomics and metabolomics, are also needed to determine the major driving factors that shape the blood microbiome in these two species.

Declarations

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Author contributions

DR, RV, FF and YSP conceived the study. All authors were responsible for the interpretation of data and critical appraisal. All authors executed the experiments and/or contributed to the experimental design and/or analyses of the results. FF and YSP drafted the manuscript with input from all authors at all stages.

Additional information

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

The sequence data that support the findings of this study are openly available in the NCBI website at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA853332. The full dataset is available at 10.6084/m9.figshare.21482325, and the Rstudio code is available at 10.6084/m9.figshare.21482355.

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Tables

Table I: Summary of the fish samples used for cmDNA analysis.

Species	Sex	N	Length (mm) mean ± SE	Weigth (g) mean ± SE
Greenland halibut	**Total**	**97**	316.2 ± 15.1	518.38 ± 72.2 [a]
	Male	19	341.2 ± 23.7	407.05 ± 67.5
	Female	32	433.0 ± 22.7	915.06 ± 125.8
	Unknown	46	224.5 ± 17.0	37.55 ± 1.6 [b]
Atlantic halibut	**Total**	**86**	762.0 ± 30.1 [c]	6 976.8 ± 879.7 [c]
	Male	55	755.1 ± 36.4 [d]	6 628.5 ± 871.0 [d]
	Female	30	760.3 ± 54.6	7 167.8 ± 1935.6
	Unknown	1	1 190	20 060

Number of samples for which the data were available: [a] n = 73, [b] n = 22, [c] n = 85 and [d] n = 54

Table II: Number of taxa in the cmDNA of the Atlantic halibut (*H. hippoglossus*) and the Greenland halibut (*R. hippoglossoides*).

Taxon	Atlantic halibut (n = 86)	Greenland halibut (n = 97)	Total
Phylum	23	29	30
Class	44	59	63
Order	88	112	121
Family	189	224	241
Genus	473	587	685

Table III: Mean relative abundance of discriminative taxa found in the cmDNA in mature and immature halibuts.

Species	Taxa	Immature mean ± SE (n = 56)	Mature mean ± SE (n = 25)	P values
Greenland halibut	*Proteobacteria*	87.3 ± 1.1 [a]	74.3 ± 2.8	< 0.001
	Firmicutes	5.1 ± 0.7	< 0.001	< 0.01
	Actinobacteria	1.6 ± 0.4	3.8 ± 0.7	< 0.001
	Bacteroidetes	3.3 ± 0.4	5.9 ± 0.9	< 0.05
	Vibrio	9.7 ± 1.2	3.3 ± 0.9	< 0.001
	Burkholderia	0.7 ± 0.3	4.5 ± 0.9	< 0.001
	Corynebacterium	0.4 ± 0.1	1.7 ± 0.5	< 0.001
	Psychrobacter	21.1 ± 2.3	10.8 ± 2.0	< 0.001
	Pseudoalteromonas	31.5 ± 2.9	13.2 ± 2.2	< 0.001
	Staphylococcus	0.6 ± 0.2	3.6 ± 1.1	< 0.001
Atlantic halibut	*Psychrobacter*	13.8 ± 2.0	7.9 ± 2.3	< 0.05
	Escherichia/Shingella	1.7 ± 1.5	9.5 ± 5.0	< 0.01

[a] Values are expressed as percentages.

Table IV: Mean relative abundance (%) of discriminative taxa found in the cmDNA of Greenland halibuts inhabiting cold and warm water.

Taxa	Cold mean ± SE (n = 25)	Warm mean ± SE (n = 72)	P values
Proteobacteria	87.2 ± 2.0[a]	79.7 ± 1.9	< 0.05
Firmicutes	4.2 ± 0.8	7.9 ± 1.0	< 0.05
Pseudoalteromonas	34.4 ± 4.9	19.9 ± 2.1	< 0.01
Vibrio	9.8 ± 1.4	5.9 ± 1.0	< 0.01

[a] Values are expressed as percentages.

Table V: Mean relative abundance (%) of the discriminative taxa found in the cmDNA of Greenland halibuts (*R. hippoglossoides*) according to the size classes.

Taxa	Class 1 mean ± SE (n = 36)	Class 2 mean ± SE (n = 22)	Class 3 mean ± SE (n = 39)	P values [b]
Proteobacteria	86.4 ± 1.4	87.5 ± 2.0	73.9 ± 3.0	< 0.01
Actinobacteria	1.8 ± 0.5	1.6 ± 0.4	3.9 ± 0.7	< 0.01
Firmicutes	5.8 ± 1.0	4.5 ± 1.0	9.3 ± 1.6	< 0.05
Vibrio	8.8 ± 1.6	10.4 ± 1.6	3.2 ± 1.0	< 0.001
Psychrobacter	27.9 ± 2.9	8.3 ± 1.4	10.8 ± 2.1	< 0.001
Acinetobacter	2.8 ± 0.7	1.4 ± 0.4	5.0 ± 1.2	< 0.05
Pseudolalteromonas	27.0 ± 2.9	37.2 ± 5.7	12.9 ± 2.2	< 0.001
Burkholderia	0.8 ± 0.4	1.8 ± 0.9	4.1 ± 0.9	< 0.001
Alcaligenes	0.1 ± 0.1	1.4 ± 1.1	0.02 ± 0.01	< 0.001
Corynebacterium	0.4 ± 0.2	0.3 ± 0.1	1.7 ± 0.5	< 0.001
Staphylococcus	0.7 ± 0.2	0.8 ± 0.3	3.7 ± 1.2	< 0.001

[a] Values are expressed as percentages.

Figures

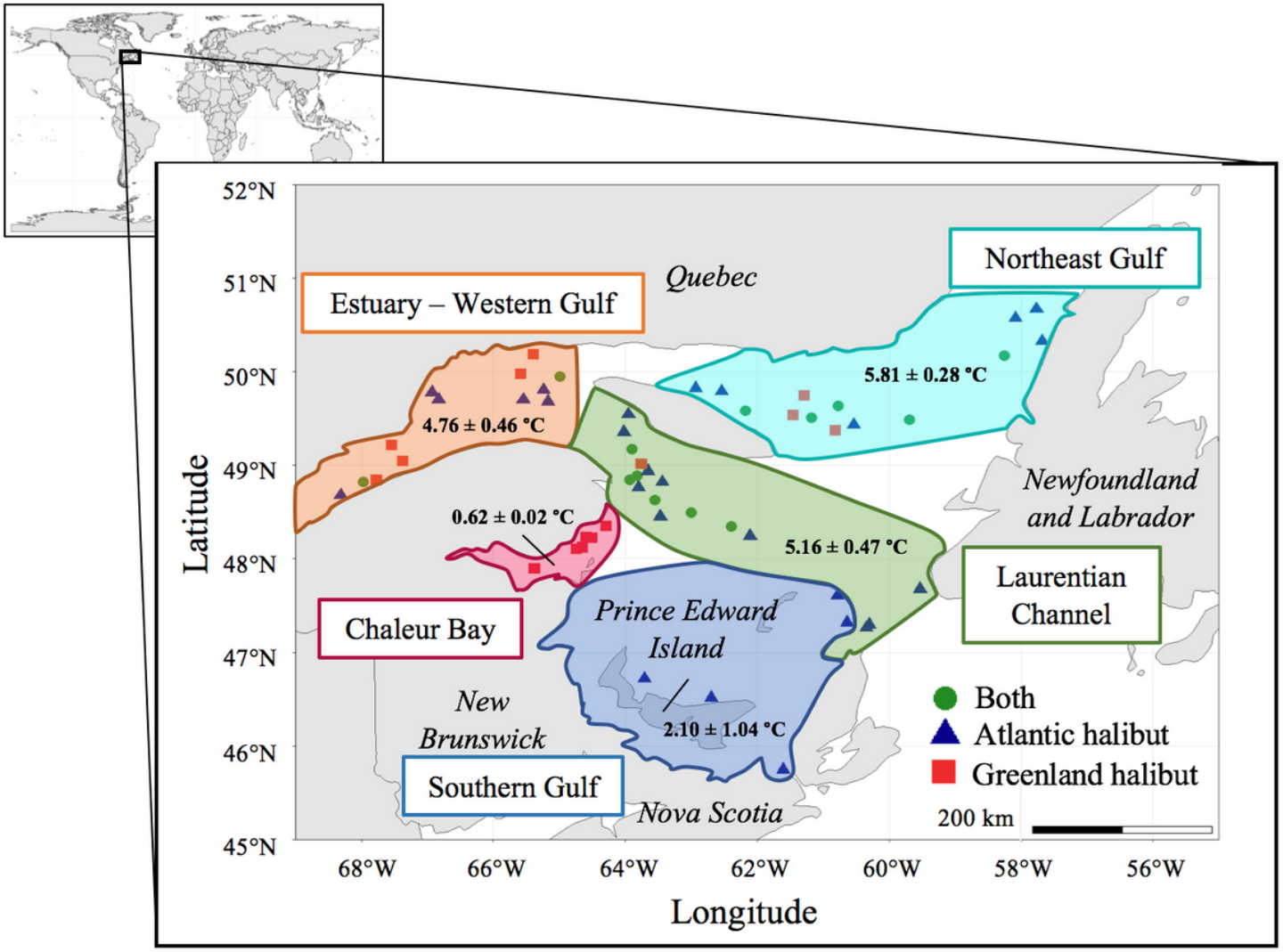

Figure 1

Map of the sample sites divided into five different areas. The regional temperature is given for each zone (mean ± SE).

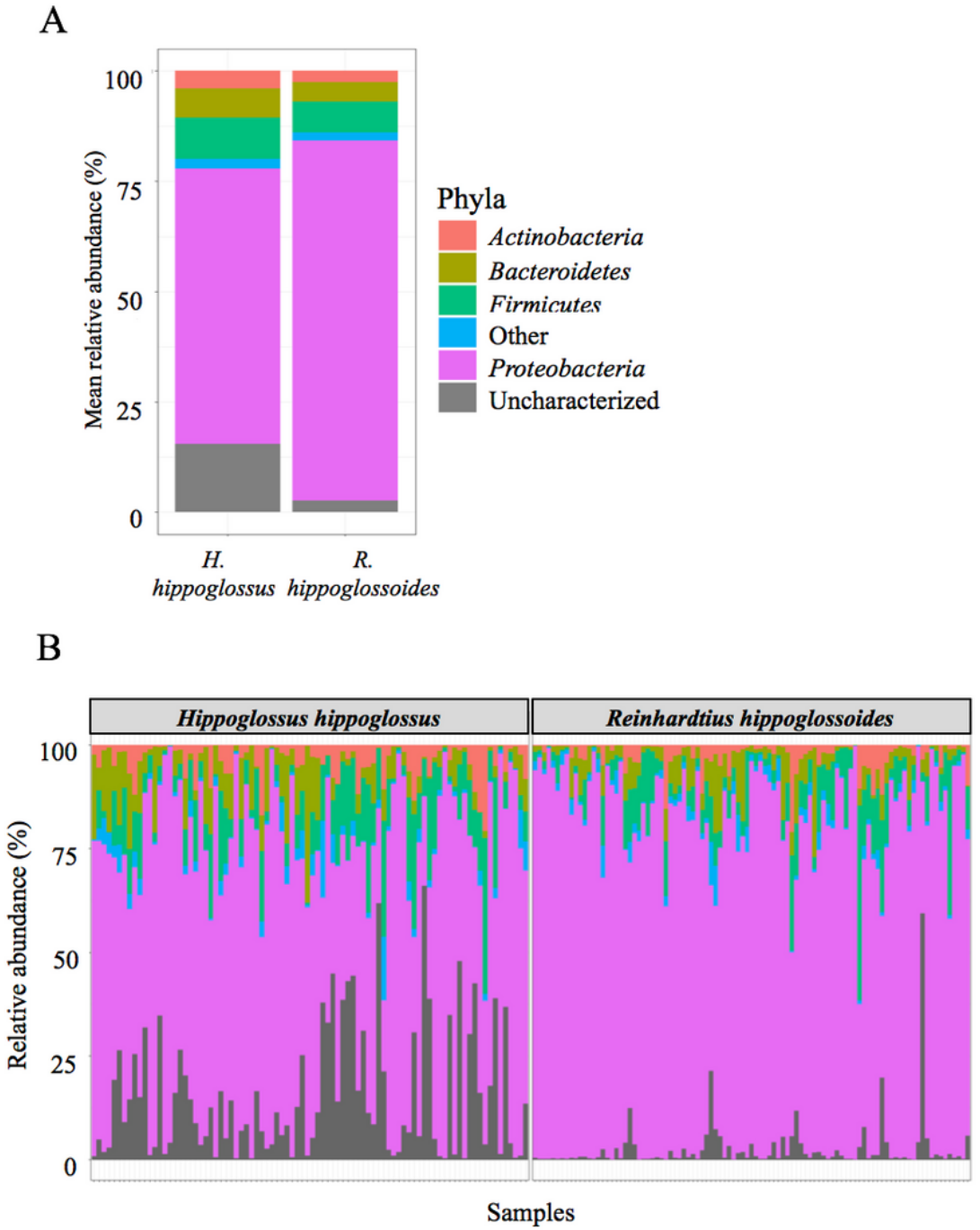

Figure 2: **Microbiome structure at the phylum level. A**. Mean relative abundance (%) of the four main phyla present in the blood microbiome of Atlantic halibut (*H. hippoglossus*) and Greenland halibut (*R. hippoglossoides*). **B**. Individual variation in the relative abundance (%) of the main phyla of Atlantic halibut (n = 86) and Greenland halibut (n = 97).

Figure 2

See image above for figure legend.

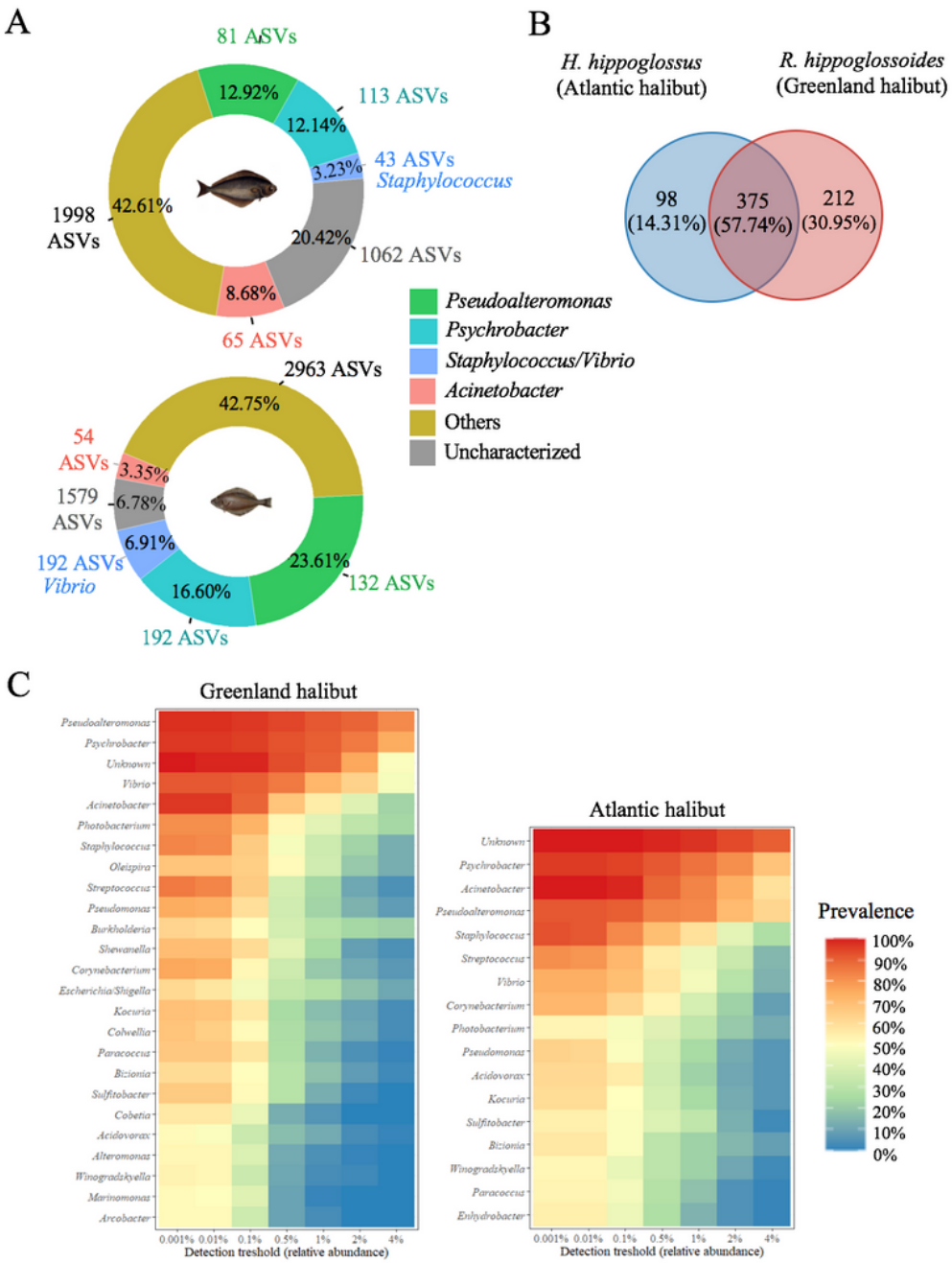

Figure 3: **Core blood microbiome analysis**. **A**. Mean relative abundance (%) of the core genera aggregated (90% prevalence) in the cmDNA of the blood microbiome of the Atlantic halibut (*H. hippoglossus*) (top) and the Greenland halibut (*R. hippoglossoides*) (bottom). The mean relative abundance is given in each pie chart, and the number of aggregated ASVs is indicated next to each pie chart. **B.** Venn diagram showing common and distinctive genera in the blood microbiome **C.** Heatmaps of the core microbiome. These heatmaps identify the most prevalent bacteria in both halibut species. Atlantic halibut, n = 86, Greenland halibut, n = 97.

Figure 3

See image above for figure legend.

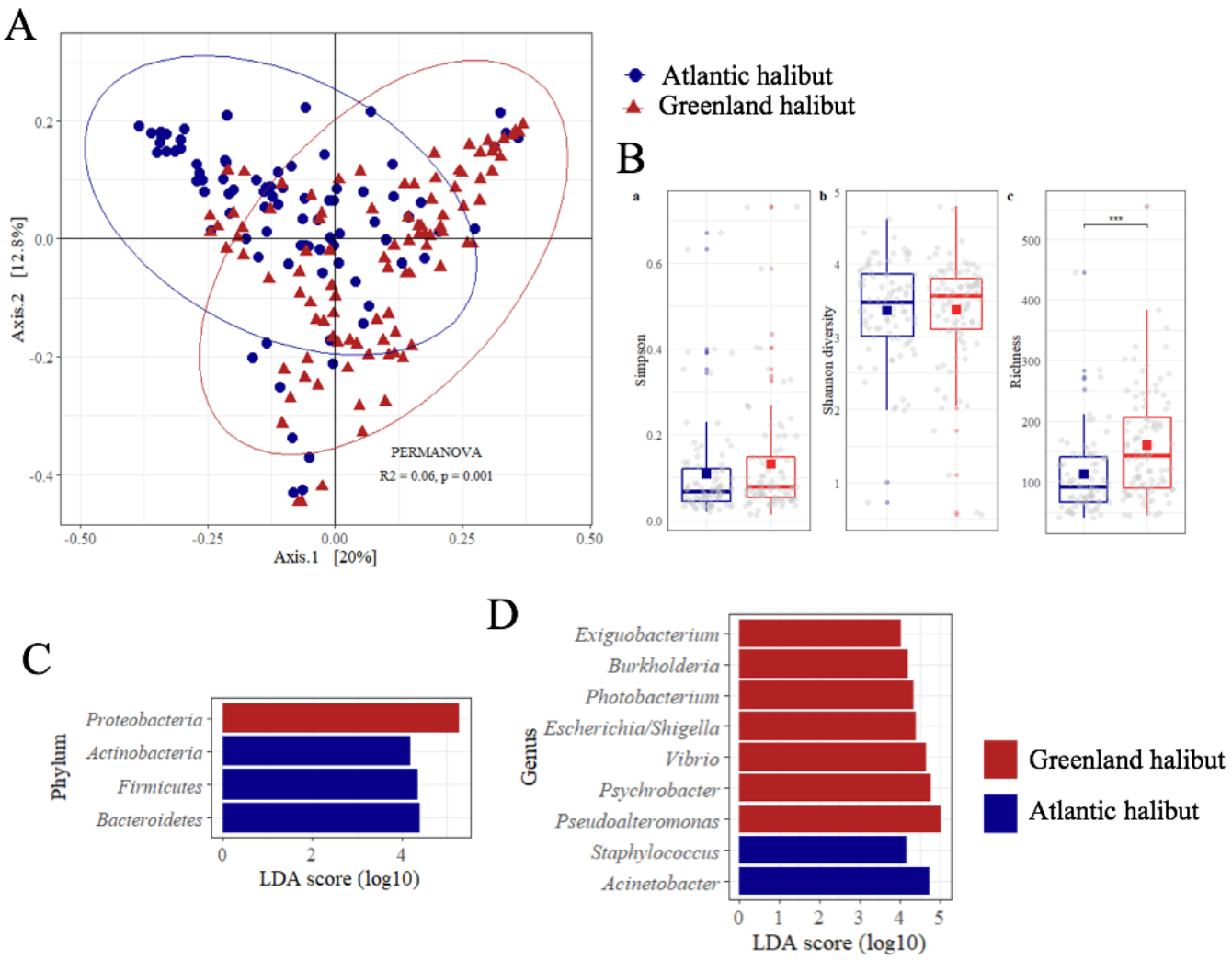

Figure 4: **Biodiversity analysis**. **A.** PCoA plot of the β-diversity of the blood microbiome based on weighted UniFrac distances. **B.** α-Diversity metrics for the cmDNA of Atlantic halibut (blue) and Greenland halibut (red). ***: $p < 0.001$. **C and D**. Discriminative taxa at the phylum and genus levels in the cmDNA of both halibut populations ($p<0.05$). Atlantic halibut, n = 86, Greenland halibut, n = 97.

Figure 4

See image above for figure legend.

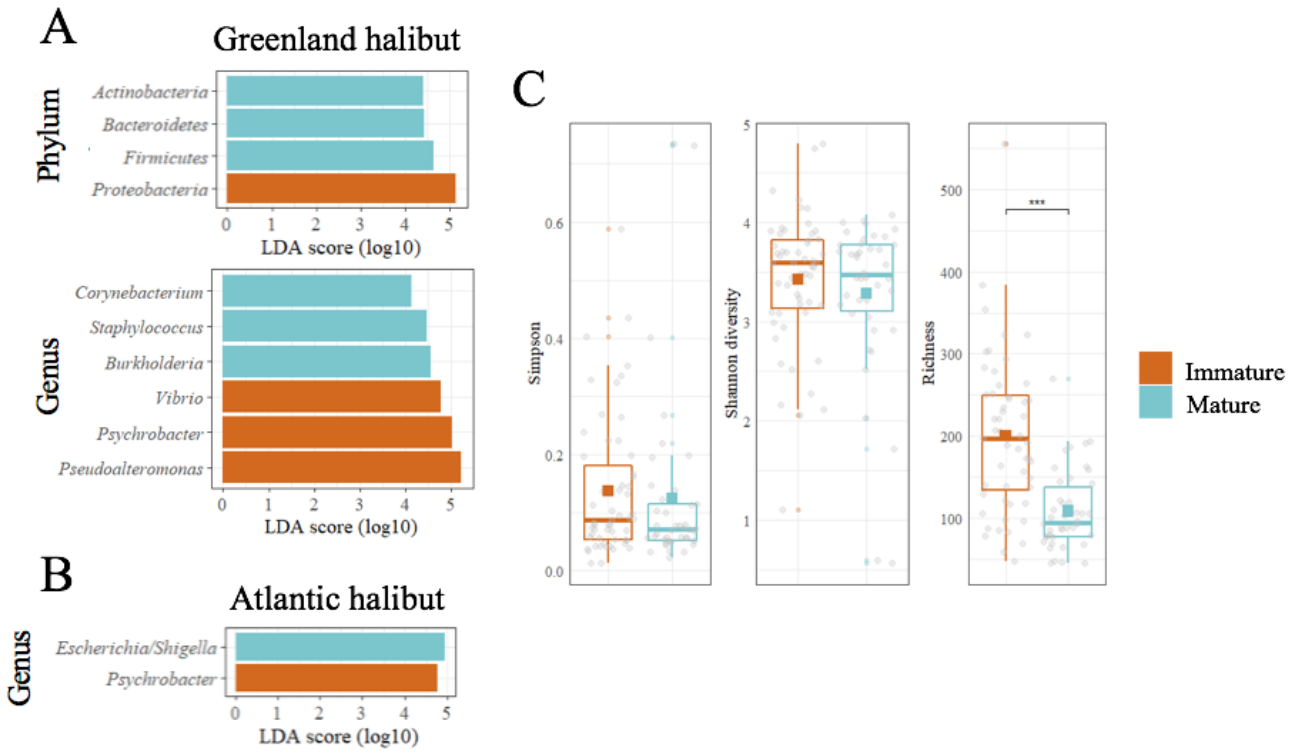

Figure 5: **Maturity analysis.** LEfSe analysis of the blood microbiome showing the significantly different taxa in mature (blue) and immature (red) fish in (**A**) Greenland halibut (Immature, n = 55, mature, n = 42) and (**B**) Atlantic halibut (Immature, n = 56, mature, n = 25). **C.** α-Diversity metrics for immature and mature Greenland halibut. ***: $p < 0.001$.

Figure 5

See image above for figure legend.

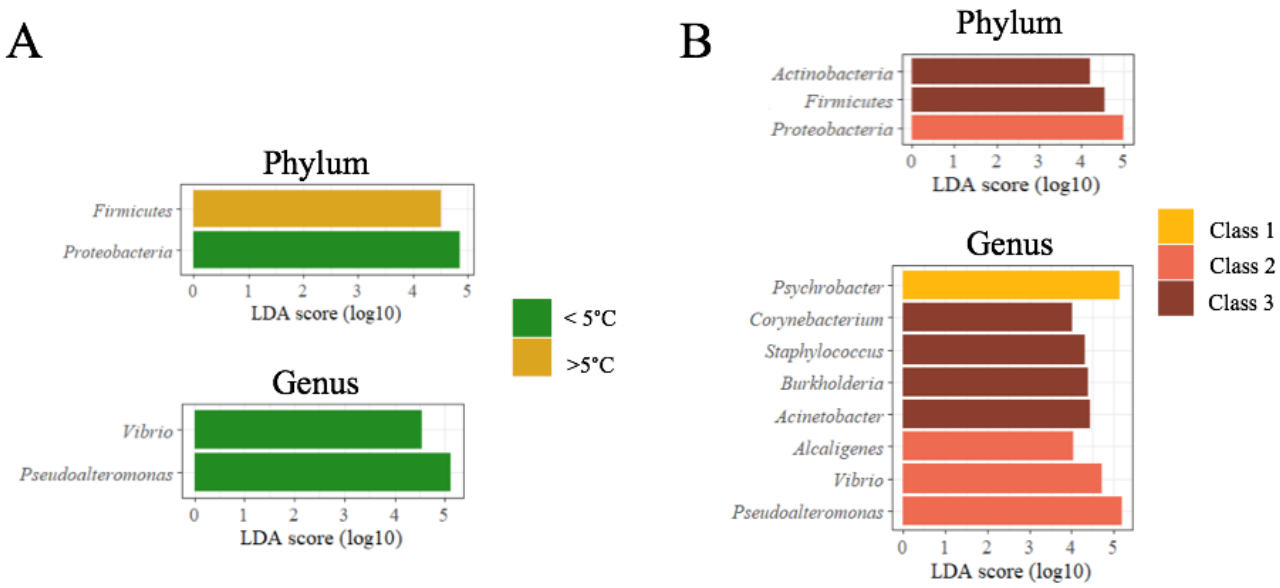

Figure 6: **Discriminant taxa associated with seawater temperature and size classes. A.** LEfSe analysis of the blood microbiome showing the significantly different taxa in Greenland halibut (**A**) inhabiting cold (n = 25) and warm (n = 72) seawater and (**B**) according to their size class (Class 1, n = 36, Class 2, n = 22, Class 3, n = 39).

Figure 6

See image above for figure legend.

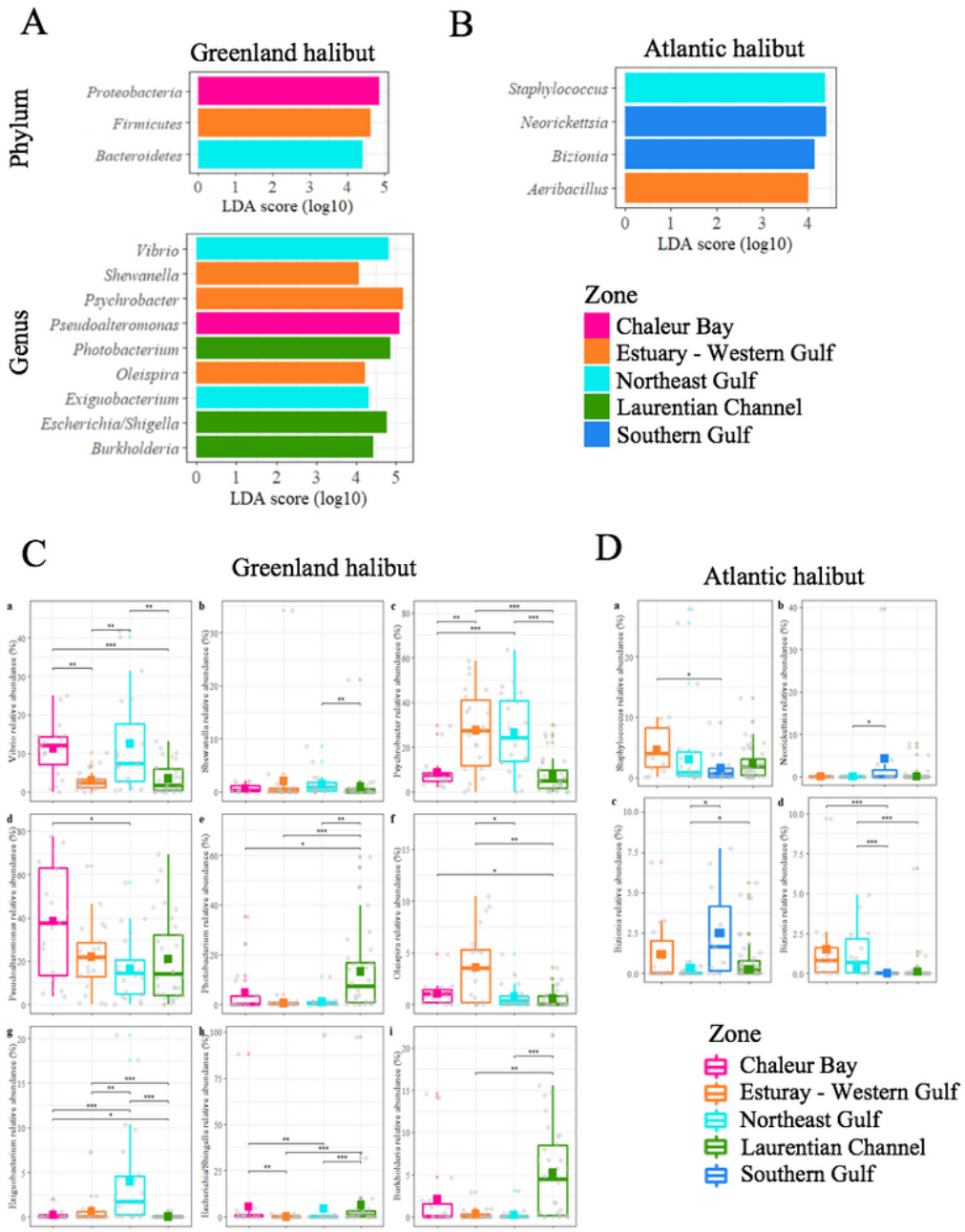

Figure 7: Spatial analysis. LEfSe analysis of the blood microbiome showing the significantly different taxa in (**A**) Greenland and (**B**) Atlantic halibut according to their localization. **C and D**. Relative abundance of the discriminative genera in Greenland and Atlantic halibut. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.00$. Greenland halibut: Estuary–Western Gulf, n = 22, Chaleur Bay, n = 19, Northeast Gulf, n = 22, Laurentian Channel, n = 34. Atlantic halibut: Estuary–Western Gulf, n = 14, Southern Gulf, n = 11, Northeast Gulf, n = 17, Laurentian Channel, n = 44.

Figure 7

See image above for figure legend.