

# Host Species and Habitat Shape Fish-associated Bacterial Communities: Phylosymbiosis between Fish and their Microbiome

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## Research Article

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# Abstract

## Background

While many studies have reported that the structure of the gut and skin microbiome is driven by both species-specific and habitat-specific factors, the relative importance of host-specific versus environmental factors in wild vertebrates remains poorly understood. The aim of this study was to determine the diversity and composition of fish skin, gut, and surrounding water bacterial communities (BCs) and assess the extent to which host habitat and phylogeny predict BC similarity. Skin swabs and gut samples from 334 fish belonging to 17 species were sampled in three Laurentian Great Lakes (LGLs) habitats (Detroit River, Lake Erie, Lake Ontario). We also collected and filtered water samples at the time of fish collection. We analyzed BC composition using 16S metabarcoding and tested for community variation.

## Results

We found that the water BC was distinct from the fish microbiome BCs, although the skin BC more closely resembled the water BC. We also found that environmental (sample location), habitat, fish diet and host species factors shape and promote divergence or convergence of the fish BCs. Since host species significantly affected both gut and skin BC (separately from host species effects), we tested for phylosymbiosis using pairwise host species phylogenetic distance versus BC dissimilarity. We found significant phylogenetic effects on BC dissimilarity, consistent with phylosymbiosis for both the fish skin and gut BCs, perhaps reflecting the longstanding co-evolutionary relationship between the host species and their microbiomes.

## Conclusions

Analyzing the gut content and skin mucus BCs across diverse fish species in complex natural ecosystems such as the LGLs provides insights into the potential for habitat and species-specific effects on the microbiome, and ultimately the health, of the host.

# Introduction

Host-associated microbiomes, specifically the bacterial community (BC) present inside the host as well as on body surfaces, influence a broad range of host immunological, evolutionary, and ecological processes (1, 2). The term “microbiome” refers to the entire microbial ecosystem, including the microorganisms (prokaryotes and eukaryotes), their genomes, and their surrounding habitats (3) and is hypothesized to have co-evolved with its host (4). Significant research effort has focused on the importance of exogenous abiotic and biotic factors (e.g., habitat, geography, microbial biodiversity, diet) and endogenous host-related factors (e.g., genetics, physiology, immunity) in driving the composition of the microbiome (5–7). At the microbial species level, deterministic (endogenous and exogenous) factors are thought to be the dominant forces shaping species composition of BCs (8–10). On the other hand, stochastic process-driven microbiome assembly (population growth rates, colonization events, extinction, and speciation) assumes that BCs species are essentially functionally equivalent (neutral), and community assembly is the result of stochastic dispersal and drift through which organisms are randomly lost and replaced (11, 12). Recent studies showed that both stochastic and deterministic processes are shaping the microbiome BC, but still a central question is the extent to which these two processes influence the host, as well as all of their associated microbes (10, 13). Despite the known effects of habitat and host-specific factors on the microbiome across host taxa, very little is known about the degree of variation in fish-associated BC diversity and composition that occurs within and among species in the wild (14). Moreover, to know how host-associated BCs may influence host phenotype, and hence contribute to evolutionary processes, quantifying the degree and nature of among-host species microbiome variation and the systematic drivers behind it seems crucial.

We expect that hosts and their microbiomes are linked eco-evolutionarily and the microbiome composition will recapitulate the phylogeny of their host; the basis of phylosymbiosis theory (15). Essentially, this predicts that hosts that are phylogenetically similar will have microbiomes that are more similar and *vice-versa* (16). Phylosymbiosis may occur through stochastic and/or deterministic processes (17), mirroring genetic evolution by drift and/or selection. However, patterns of phylosymbiosis are also expected to be affected by exposure to habitat microbiome(s) (18). Although most studies in which phylosymbiosis has been identified have focused on microbes inhabiting internal organs, such as the gastrointestinal tract (15), recent work suggests that external host microbiomes (e.g., skin) can also exhibit a phylosymbiosis signal (19). In expanding the range of studied vertebrate microbiomes, questions about the range of environmental, ecological and evolutionary factors that shape gut and skin microbiomes (or more specifically, BCs), and the functions of those communities, still remained unanswered. For example, similar gut BCs are found among phylogenetically related mammals but also among unrelated mammal species with similar diets (20–22).

Most host-microbial interaction studies are mainly focused on mammalian species, with fewer studies focussed on other species (23). Teleosts encompass over one half of vertebrate diversity (24) and are one of the most successful groups of vertebrates on Earth (25). Teleosts are represented by more than 32 000 species, originated over 600 million years ago and exhibit a variety of physiologies, natural/life histories and ecologies (26). However, their success and species radiation would not have been possible without the help of their microbiome (27). Additionally, their long history of co-evolution and symbiotic relationships with microbes (compared to mammals which evolved 160 million years ago (21)) make them good candidates to study host-microbial interactions. However, while most published studies of fish microbiomes include the gut microbiome, few studies have included other key microbiome habitats such as skin (28, 29). The role the skin microbiome in host health is under-studied, and, importantly for this study, the skin microbiome may be thus under separate selective pressures than the gut microbiome (14).

The Laurentian Great Lakes (LGLs) in North America form the largest freshwater ecosystem on the planet and have provided valuable ecosystem services for humans for centuries (30). The LGLs and their associated drainages include diverse ecosystems, complex trophic interactions, and mixed watersheds including forested, wetland, agricultural and urban areas. The LGLs thus represent a powerful natural ecological laboratory to study important questions concerning microbial ecology and host-microbe interactions and co-evolution. While much work has been done on characterizing microbial communities in LGLs themselves, there is a critical need to examine the roles of host species and habitat on the microbiome of fish at the fish community level. The fish and microbial communities of LGLs provide a novel opportunity to study the intra- and interspecific divergence of host-associated intestinal and skin BCs among the diverse species of the fish communities.

The aim of this study was to characterize fish microbiome BCs among a variety of host species across three locations to: (i) identify patterns of fish-associated BCs, (ii) quantify the similarity among water BCs and those of the fishes, and (iii) determine the extent to which BCs among fish follow a pattern of phylosymbiosis. Our first hypothesis is that host-related deterministic processes (host-based selection pressures on BC composition) are the main drivers of BC composition variation, and hence most BC composition variation will be found among species. Our second hypothesis is that the water BC will be distinct from the host-associated BC, and the gut BC will also be different from the skin BC, but more similar to the water BC than the gut. Finally, we predict that the gut microbiome will be more strongly correlated with host phylogeny than the skin microbiome, as the skin BC will be affected by the local environmental microbiome more strongly. Characterizing intraspecific variation in fish microbiomes will help managers, particularly in aquaculture settings, effectively manipulate gut and skin BCs to promote animal health and well being. On the other hand, such information may provide wild fish population managers tools to assess fish stock status and health. More broadly, demonstrating the possible existence of phylosymbiosis in fish and their microbiomes will provide insight on factors governing the microbial community associated with fish and their co-evolutionary dynamics with teleosts in general.

## Materials And Methods

### Study sites

**Detroit River:** The Detroit River of the LGLs is a 51 km channel that comprises the lower portion of the Huron-Erie Corridor, connecting Lake St. Clair to Lake Erie (31). Samples were collected around Fighting Island (from 42°10'56.5"N 83°06'39.9"W up to 42°14'04.2"N 83°06'32.1"W) from July 17 to August 29, 2018.

**Lake Erie:** Samples came from the western basin of Lake Erie, a shallow (mean 6m), warm, and productive basin (area = 26 km<sup>2</sup>) fed by the Detroit River and several smaller tributaries draining agricultural watersheds. Samples were collected on October 18, 2018

**Lake Ontario:** Fish were collected from the Bay of Quinte and the eastern basin of Lake Ontario from July 31 to August 1, 2018. The Bay of Quinte is a large (254 km<sup>2</sup>), Z-shaped embayment with a history of nutrient and anthropogenic stress that feeds into the eastern basin and the upper St Lawrence River. The eastern basin is a mildly eutrophic, bathymetrically complex outlet basin of Lake Ontario with depths averaging ~20m.

### Sample collection

**Detroit River:** Fish were captured using a single anode boat electrofisher (Smith-Root 5.0 GPP) set to use pulsed DC current at 60 Hz using between 30%– 60% of the range to maintain a current of 6–8 A. All fish were euthanized with an overdose of tricaine methanesulfonate (MS222) following the protocols of the Ontario Ministry of Northern Development, Mines, Natural Resources and Forestry (NDMNR) and Canadian Council on Animal Care policies. The skin swabs for all fishes were immediately taken after capture by gently rubbing a sterile cotton swab over ~ 50% of the total surface on the right side of each fish. Dorsal, ventral, and pectoral fin areas were swabbed for larger

fish. Swab samples were placed into 2mL tubes and were stored on ice and transported to the Great Lakes Institute for Environmental Research (GLIER) at the University of Windsor and immediately frozen at -20°C. The whole fish were transported on ice with the swabs to GLIER and within 2-4 hours after capture the fish were dissected, and gut content samples were collected and stored frozen at -20°C. Water samples (500 mL) were collected at the sampling sites and transported to GLIER on ice for filtration. Water samples were filtered using 0.22-µm pore size, 47 mm diameter polycarbonate membranes (Isopore™, Millipore, MA) and stored at -20°C until DNA extraction.

Lake Erie: Fish were captured using bottom-set, graded mesh (32- to 152-mm) stretch monofilament gillnets. After capture, all fish were euthanized in compliance with the protocols of the NDMNRF and Canadian Council on Animal Care policies. The skin, fish and water sampling, as well as the transportation and storage of the samples were the same as for Detroit River.

Lake Ontario: Fish were captured using bottom-set, graded mesh (38-152 mm) stretch monofilament gillnets. Upon capture, all fish were euthanized following the protocol described for Detroit River and Lake Erie. Skin swab samples and gut tissue (foregut, midgut, and hindgut) with content were taken at the Glenora Fisheries Station, Ontario, Canada. Samples were stored in a 50 mL falcon tubes filled with 45 mL of a high salt solution (700 g/L Ammonium Sulfate, 25 mM Sodium Citrate, 20 mM Ethylenediaminetetraacetic acid, pH 5.2) for 48 hours to let the salts penetrate the samples (swabs, and gut content), and then stored at -20 °C until DNA extraction. Water samples (500 mL) were collected and filtered at the Glenora Fisheries Station by using 0.22-µm filters pore size, 47 mm diameter and the filters kept in high salt solution until delivered to GLIER. All samples were stored at -20°C until DNA extraction.

### Sample information

A total of 334 fish from Detroit River (n= 98, 29%), Lake Erie (n= 90, 27%), and Lake Ontario (n=146, 44%) representing 17 different species were collected for microbiome characterization. The fish species included herbivores, invertivores, piscivore, planktivores, planktivore/invertivore, invertivores/carnivores, invertivores/detritivores, and invertivores/herbivores (Table 1; Supplementary Table S1) (32, 33). For each of the 334 fish, fork length and total weight were recorded. Gut samples were taken after carefully dissecting the fish with a new razor blade or sterilized scissors to isolate a section comprising midgut and hindgut with both tissue and gut content. For Detroit River, Lake Erie, and Lake Ontario (based on sampling locations) a total of 4, 3, and 5 water samples were collected at the site of capture, respectively.

### DNA Extraction, Library Construction and Sequencing

DNA from all samples were extracted using a sucrose lysis buffer protocol as previously described (34). The V5 (787 F-acctgctgccc-ATTAGATACCCNGGTAG) and V6 (1046 R-acgccaccgagc-CGACAGCCATGCANCACT) variable regions of the 16S rRNA were selected for PCR amplification. The first and second PCR conditions were same as previously published methods (34). Briefly, The PCR protocol for the first round PCR consisted of: 95 °C for 3 min followed by 28 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 m, and a final step at 72 °C for 10 m. For each 96 well PCR plate, one negative control consisting of PCR mix with ultra-pure water instead of DNA template, was included. After the first-round PCR amplification, the results were verified by visualizing amplicons on an agarose gel. After checking and verifying first-round PCR products, the PCR was purified using Sera-Mag Magnetic Beads (GE, Healthcare Life Science, UK). A short-cycle second round PCR was conducted on the purified PCR products to ligate the adaptor and barcode (10 -12 bp) sequences necessary for sample identification and sequencing. The second PCR was set at 95 °C for 3 min, then 8 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 7 min. The second round PCR amplifications were visualized on an agarose gel and amplicons were combined in proportions based on their estimated concentration (between 1- 5 µL for samples with strong clear (1 µL) to faint bands (5 µL)). Subsequently, the combined samples were gel extracted from an agarose gel and cleaned and purified using QIAquick Gel Extraction Kit (QIAGEN, Toronto, ON, Canada). In total 299 (89%), 330 (99%), and 10 (83%) samples for gut, skin swab and water were amplified successfully and included in the sequencing library. We also included eight PCR blanks (one for each 96 PCR plate) in our library. The concentration of purified PCR product mix (library) was measured on an Agilent 2100 Bioanalyzer with a High Sensitivity DNA chip (Agilent Technologies, Mississauga, ON, Canada). The library concentration was then diluted to 60 pmol·µL<sup>-1</sup> and sequenced on an Ion S5™ sequencing system using the Ion S5™ sequencing reagents and an Ion 530™ Chip (Thermo Fisher Scientific, ON, Canada).

### Processing of 16S sequences

Two FASTQ files (one for gut and water samples and one for swab samples) were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME2-2020.11) platform (35). The FASTQ sequence files were demultiplexed using the *cutadapt demux-single* command to remove sample barcode and primer sequences. Additionally, *cutadapt trim-single* was used to identify and remove the sequencing adapters for the demultiplexed data (36). The DADA2 pipeline (*dada2 denoise-pyro*) was used to denoise single-end sequences, dereplicate and filter chimeras, followed by Amplicon Sequence Variant (ASV) picking (37). Chimeric sequences were removed using the

*removeBimeraDenovo* function with the “consensus” method using the default values, except the read truncation length was set to 270 (*p-trunc-len 270*). The two ASV tables and representative sequences were merged using *feature-table merge* and *feature-table merge-seqs*, respectively. Taxonomic classification was performed using the *feature-classifier* plugin (38) and the SILVA 138-99 reference database (39). This plugin supports taxonomic classification of features using the Naive Bayes method. All ASVs were aligned with *mafft* (40) (via *phylogeny align-to-tree-mafft-fasttree* command) and used to construct a phylogeny with *fasttree* (41). After quality control, chimera removal and combining the two feature tables, the table was summarized with the *feature-table summarize* command. A total of 18 147 574 sequences were obtained for 647 samples (299 gut samples, 10 water samples, 330 skin samples plus eight negative controls). The eight negative controls had zero to 10 sequence reads with no consistent taxa present. The *decontam* (42) (version 1.8.0) in the R package were used to identify the blank sample ASVs as possible contaminants in our sample BCs; however, none of the blank sample ASVs were identified as a contaminant. Thus, the negative controls were excluded from the rest of the study and we assumed contamination was not an issue for our data set.

We used the *taxa filter-table*, to remove ASVs related to mitochondria, chloroplast and eukaryote sequences (3% of total sequence). We also removed “Unassigned” ASVs (13%), as well as Bacteria and Archaea ASVs without phylum assignment (1%), resulting in a total of 14 990 847 sequences remaining. The ASV table was rarefied to 3000 reads per sample for the alpha and beta diversity estimation because most of the rarefaction curves plateaued at ~3000 reads. Samples with fewer than 3000 reads were deleted. These deleted samples included three gut and six swab samples (Lake Trout (*Salvelinus namaycush*) (1 gut, 2 swab samples), White Perch (*Morone americana*) (1 gut), White Bass (*Morone chrysops*) (1 gut), Blacknose Shiner (*Notropis heterolepis*) (1 swab), Pumpkinseed (*Lepomis gibbosus*) (1 swab), White Sucker (*Catostomus commersonii*) (1 swab), Freshwater Drum (*Aplodinotus grunniens*) (1 swab)). This decreased the total number of samples to 630 samples (296 gut, 324 swab and 10 water samples) (Table 1). ASVs were retained for further analysis only if they had at least 10 sequence reads in at least two samples. So, the final analysis included 13 884 500 reads (93% of reads were retained) and 6 597 ASVs (15% of total ASVs (43 763)).

#### **BC Alpha and Beta Diversity:**

BC alpha diversity indices were calculated for each sample using QIIME alpha diversity alpha command. The calculated alpha diversity indices were Chao1 (a metric for species richness), and Faith’s phylogenetic diversity (PD) (a metric that incorporates both species richness and species evenness). We estimated beta diversity as the Bray–Curtis dissimilarity matrix among all samples. The rarefied ASV table was used for all subsequent analyses, unless stated. Raw data are available at the Sequence Read Archive of NCBI with PRJNA701818 BioProject accession number.

Table 1: Summary of Great Lakes fish species gut, skin and water samples. We provide a description of taxonomy and feeding guild of the host species as well as where the fish were sampled. The number of samples included in our analyses is shown for both skin and gut samples (and total number for each fish species) after quality filtering and rarefaction.

Genus	Species	Fish species (common name)	Feeding	Habitat	Detroit River		Lake Erie		Lake Ontario		T*
					G*	S*	G	S	G	S	
<i>Alosa</i>	<i>pseudoharengus</i>	Alewife	Planktivore	Pelagic	-	-	-	-	20	21	41
<i>Aplodinotus</i>	<i>grunniens</i>	Freshwater Drum	Invertivore	Benthic	1	5	17	16	10	10	59
<i>Ambloplites</i>	<i>rupestris</i>	Rock Bass	Invertivore	Benthopelagic	3	7	-	-	3	3	16
<i>Catostomus</i>	<i>commersonii</i>	White Sucker	Invertivore/detritivore	Benthic	5	9	-	-	2	2	18
<i>Cyprinella</i>	<i>spiloptera</i>	Spotfin Shiner	Invertivore/herbivore	Benthopelagic	12	11	-	-	-	-	23
<i>Dorosoma</i>	<i>cepedianum</i>	American Gizzard Shad	Herbivore	Pelagic	9	10	-	-	2	2	23
<i>Labidesthes</i>	<i>sicculus</i>	Brook Silverside	Planktivore / invertivore	Pelagic	7	7	-	-	-	-	14
<i>Lepomis</i>	<i>gibbosus</i>	Pumpkinseed	Invertivore	Benthopelagic	5	10	-	-	5	5	25
<i>Morone</i>	<i>americana</i>	White Perch	Invertivore/carnivores	Benthopelagic	-	-	21	21	11	12	65
<i>Morone</i>	<i>chrysops</i>	White Bass	Invertivore	Benthopelagic	-	-	10	10	10	11	41
<i>Neogobius</i>	<i>melanostomus</i>	Round Goby	Invertivore	Benthic	-	-	8	8	16	16	48
<i>Notropis</i>	<i>atherinoides</i>	Emerald Shiner	Planktivore	Benthopelagic	5	15	-	-	-	-	20
<i>Notropis</i>	<i>heterolepis</i>	Blacknose Shiner	Invertivore/herbivore	Benthopelagic	11	8	-	-	-	-	19
<i>Perca</i>	<i>flavescens</i>	Yellow Perch	Invertivore	Benthopelagic	10	10	15	15	15	16	81
<i>Salmo</i>	<i>trutta</i>	Brown Trout	Piscivore	Benthopelagic	-	-	-	-	16	16	32
<i>Salvelinus</i>	<i>namaycush</i>	Lake Trout	Piscivore	Benthopelagic	-	-	-	-	22	22	44
<i>Sander</i>	<i>vitreus</i>	Walleye	piscivore	Benthopelagic	-	-	19	19	6	7	51
-	-	Water samples	-		3	-	2	-	5	-	10

\*Abbreviation: G (Gut), S (Skin), T (Total number of fish)

### Statistical analysis of sequence variants

**Fish versus Environmental (Water) BCs:** To test for the effect of the environmental (water) BCs on gut and skin sample BCs, taxonomical compositions of the BCs from the different sample types (gut, skin and water) were visualized using stacked barplots of the relative abundance of the bacteria at the phylum and family level with the online tool MicrobiomeAnalyst (43). Subsequently, a Non-metric multidimensional scaling (NMDS) plot using the Bray Curtis distance matrix was generated using *vegan* (v.2.5-7) (44) and *ggplot2* (v.3.3.5;(45) packages in R (v.4.1.0; (46) to visualize clustering among the sample types (skin, gut, and water). We then performed permutational analyses of variance (PERMANOVA) using *adonis2* in the *vegan* (v.2.5-7) (44) package in R (46) to test for significant differences in beta diversity among the sample types (gut vs water, as well as skin vs water samples). Finally, differences in alpha diversity (species richness and evenness (Chao1, PD)) among the different sample types (gut vs water, as well as skin vs water samples) at all locations combined as well as within each location separately were tested using the Kruskal-Wallis (KW) rank test, followed by a *post hoc* Dunn test with Bonferroni corrected *P* values in SPSS (IBM SPSS 25).

**Fish Gut versus Skin BCs:** Taxonomic composition of the BCs of gut and skin samples from seventeen fish species sampled at three locations (Detroit River, Lake Erie, and Lake Ontario) were visualized using stacked barplots of the relative abundance of the bacteria at the family level using *phyloseq* (47) and *ggplot2* (45) packages built on R (46). Bacteria families with relative abundance less than 10% (ranging between 0-9.99%) in all samples were combined and presented as "Family <10 percent" in barplots. To test for the differential

abundance of bacterial taxa between the gut and skin microbiomes, the ASV table data were aggregated to the family level in R (v.4.1.0) using phyloseq package (47). Subsequently, the non-normalized ASV table was used for the negative binomial Wald test in DESeq2 (48). We used the default DESeq2 settings with negative binomial generalized linear model (GLM) fitting with Wald significance tests. *P*-values were adjusted for multiple testing using the Benjamini Hochberg false discovery rate correction (FDR) (49). Bacterial taxa showing differential abundance were defined with the thresholds of  $FDR < 0.05$  and  $|\log_2 \text{Fold Change (FC)}| > 2$ . Differences in alpha diversity (Chao1, PD) between gut and skin samples was statistically tested using the KW rank test (as described in Fish versus Environmental (Water) BCs section above). To test for significant beta diversity differences between gut and skin samples we used PERMANOVAs analysis with *adonis2* in *vegan* (v.2.5-7;) (44) package in R (46).

**Endogenous and Exogenous Factor Analyses:** Gut and skin samples clustering based on location and fish species were visualized using an NMDS plot. Observed differences were assessed for significance with PERMANOVA analyses using *adonis2* in *vegan* (v.2.5-7) (44); however, we only included samples where fish species occurred in at least two of the three locations. We further explored the role of host fish species and sampling location on the skin and gut BCs using linear mixed-model analyses. We first quantified beta diversity in the gut and skin BCs using a Principal Coordinates Analysis in R (v.4.1.0) and retained the first five PCoA axes values (gut sample PCoA factors retained = eigenvalue >2, percent of variation > 6%; skin sample PCoA factors retained =: eigenvalue >5, percent of variation > 3%). We used Chao1 and PD (as described above) to quantify alpha diversity. To determine the effect of fish species (and body weight) and (location) factors on alpha (Chao1, PD) and beta (PCoAs 1 to 5) diversity indices for gut and skin samples we built two linear mixed models (LMM) in R (v.4.1.0) using the *lme4* (50) package: the first model included location and fish species and fish species x location interaction as fixed factors, and included fish weight as a random factor. We  $\log_{10}$  transformed weight, Chao1 and PD to meet the normality and heteroscedasticity assumptions of LMMs. Weight was found to be not significant for all analyses and was dropped from all subsequent analyses.

Given that we found evidence for significant species, location and interaction effects on both gut and skin microbiome BC alpha and beta diversity, we included further analyses of possible mechanisms driving those effects. Specifically, the location effect may reflect habitat preferences among the 17 fish species sampled and the species effect may reflect diet preferences among the sampled species. To explore diet and habitat preference effects on gut and skin BCs, we used a second LMM with fish species, habitat and diet as fixed factors and location as a random factor to test for effects on skin and gut microbiome Chao1 and PD (alpha diversity) as well as PCoA1-5 (measures of beta diversity).

### Host-microbiome BC phylosymbiosis

To test if skin and gut microbiome composition were linked with host phylogeny, cytochrome c oxidase I (*COX1*) and cytochrome b (*cytb*) sequences for the 17 host fish species were downloaded from the NCBI website (<https://www.ncbi.nlm.nih.gov/gene>) and combined to create an artificial concatenated sequence. The concatenated sequences were aligned using MUSCLE (51) on the CIPRES Science Gateway v.3.1 (52). Subsequently, pair-wise phylogenetic distances between the species were calculated using the Kimura two-parameter model of substitution in MEGA-X (version 10.2.6) (53). Finally, to evaluate the effect of host phylogeny on microbiome dissimilarity (phylosymbiosis), we performed Mantel tests (999 permutations) in R (version 4.0.) using the *vegan* (V2.5-7) package (54) to compare the bacterial community Bray-Curtis dissimilarity matrixes (skin and gut) and fish species phylogenetic distance matrix. To do so, Bray-Curtis dissimilarity was calculated across all samples within each fish species.

## Results

**Fish versus Environmental (Water) BCs:** Taxonomic composition of the skin, gut and water BCs were distinct; however, the water BC was most divergent. Across all fish species, the fish microbiomes were dominated at the phylum level by Proteobacteria (78 % (skin), 56% (gut)), Fusobacteriota (6% (skin), 19% (gut)), and Firmicutes (7% (skin), 18 % (gut)). However, the water sample taxa showed a different set of common taxa, with only Proteobacteria in common among the fish BC taxa (Proteobacteria (38%), Actinobacteriota (27%), Bacteroidota (18%)) (Fig. S1). At the family level, the fish microbiomes were dominated by members of *Aeromonadaceae* (gut (21%), skin (19%)), *Fusobacteriaceae* (gut (19%), skin (6%)), *Enterobacteriaceae* (gut (17%), skin (17%)), and *Moraxellaceae* (gut (1%), skin (20%)) (Fig. S2). However, at the family level, the water microbiome was dominated by *Comamonadaceae* (23%), *Sporichthyaceae* (19%), and *Chitinophagaceae* (5%) (Fig. S2).

Measures of alpha diversity (Chao1, PD) showed higher diversity in the water BC than in the fish gut and skin BCs (Chao1: KW 65,  $P < 0.0001$ ; PD: KW 74,  $P < 0.0001$ ) (Fig. S3, Table 2). The *post-hoc* pairwise comparisons revealed that when water samples were compared against gut and skin samples, gut sample BCs were more divergent (Chao1: test statistic -292, adj  $P < 2.00E-06$ ; PD: test statistic -316, adj  $P < 1.97E-07$ ) than the skin BCs (Chao1: test statistic -192, adj  $P < 2.00E-03$ ; PD: test statistic -208, adj  $P < 1.00E-03$ ) (Fig. S3, Table 2). We also tested for differences between gut vs water, and skin vs water separately within each location. The differences between gut vs water compared to skin vs water were more pronounced when we performed the analysis within each location (Table 2).

The NMDS plot also showed clear separation between the fish microbiome BCs and the water BCs (Fig. 1). The gut and skin BCs showed considerable overlap in the NMDS; however, the water BC clustered separated from the fish BCs, indicative of different community composition (Fig. 1). PERMANOVA analyses confirmed the statistical significance of the NMDS clusters (PERMANOVA pseudo-F: 12.8,  $P$  value  $< 0.001$ ). Subsequently, individual PERMANOVAs for gut-water and skin-water comparisons showed significant effects for gut BCs (t-value: 3.02; p-value  $< 0.001$ ) and skin BCs (F-value: 3.01; p-value  $< 0.001$ ) compared to water BCs.

Table 2: Results of the Kruskal-Wallis H test followed by a *post hoc* Dunn test testing differences in alpha diversity indices (Chao1, and Faith's phylogenetic diversity (PD)) for gut, skin, and water samples among all locations followed by separate tests within each location.

Variables	Diversity index	<i>Post-hoc</i> Pairwise Comparisons	Test Statistic	Std. Error	Adj. Sig. <sup>a</sup>	d	Kruskal-Wallis H
All locations	Chao1	gut-skin	-99	14.6	2.94E-11	2	63 ***b
		gut-water	-292	58.5	2.00E-06		
		skin-water	-192	58.4	2.00E-03		
	PD	gut-skin	-108	14.6	4.69E-13	2	74 ***
		gut-water	-316	58.5	1.97E-07		
		skin-water	-208	58.4	1.00E-03		
Lake Ontario	Chao1	gut-skin	-36	9.8	7.00E-04	2	23 ***
		gut-water	-134	37.7	1.00E-03		
		skin-water	-98	37.7	0.02		
	PD	gut-skin	-62	9.8	7.14E-10	2	52 ***
		gut-water	-160	37.7	5.00E-05		
		skin-water	-98	37.7	0.02		
Lake Erie	Chao1	gut-skin	-67	7	0.00E+00	2	76 ***
		gut-water	-85	37	0.06		
		skin-water	-18.	37	1		
	PD	gut-skin	-72	7	0.00E+00	2	88 ***
		gut-water	-97	37	0.02		
		skin-water	-24	37	1		
Detroit River	Chao1	gut-skin	7	7	1	2	8*
		gut-water	-76	27	0.01		
		skin-water	-69	27	0.03		
	PD	gut-skin	17	7	0.04	2	13**
		gut-water	-84	27	7.00E-03		
		skin-water	-66	27	0.01		

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

b. Significance codes:  $0.01 < P \leq 0.05^*$ ,  $0.001 < P \leq 0.01^{**}$ ,  $P \leq 0.001^{***}$

Fish Gut versus Skin BCs: Taxonomic analysis of the BCs of skin and gut showed that Proteobacteria (Fig. S1), and specifically members of the *Aeromonadaceae*, and *Enterobacteriaceae* families generally dominated the fish microbiomes (Fig. 2, 3, and S2). However, gut and skin samples exhibited different BCs (Fig. 2, 3). Differential abundance analysis at the family level was used to acquire more specific insight into differences in microbiome BC composition between the gut and skin microbiomes. Comparing bacterial abundance data at the family level between skin BC and gut BC identified 37 bacteria families that had statistically significant differences (Table S2). For example, while members of the *Deinococcaceae*, *Exiguobacteraceae*, and *Moraxellaceae* families were at high abundance in skin samples, they were rare in the gut sample BCs. On the other hand, *Microbacteriaceae* and *Lachnospiraceae* were at higher abundance in the gut BCs (Table S2).

Moreover, the alpha diversity comparisons between gut and skin samples showed that skin samples had higher diversity relative to gut samples (Chao1: test statistic -99, adj P < 2.94E-11; PD: test statistic -108, adj P < 4.69E-13) (Table 2, Fig. S3). Although gut and skin samples showed considerable overlap in the NMDS plot (Fig. 1), our PERMANOVA analysis showed that gut and skin samples had significantly different BCs based on Bray-Curtis dissimilarity (t-value: 4.08; p-value < 0.001)

Endogenous and Exogenous Factor Analyses: The BC composition in the gut and skin samples of different fish species sampled at different locations often showed high levels of variation among and within host species and among locations, highlighting the potential effect of location and fish species on fish BCs (Fig. 2, 3). Furthermore, NMDS analysis showed that BC composition clustered based on the host fish location (Lake Erie, Lake Ontario, Detroit River) (Fig. 4A-B) and host fish species within the location for both gut and skin BCs (Fig. 4C-D). PERMANOVA analyses supported those clustering patterns and showed highly significant effects of location, host fish species and the interaction between host fish species and location on both the gut and skin BCs (Table 3).

We used LMM analyses to further explore the effects of location, fish species, and their interactions on alpha (Chao1, PD) and beta diversity (PCoA 1- 5) measures for the gut and skin BCs. LMM results showed that in general for alpha diversity indices, fish species effect was stronger for gut samples than skin samples, while skin samples showed stronger location and species-by-location interaction effects (Table 4). Moreover, location, fish species and their interaction generally had strongly significant effects on beta diversity indices (PCoAs) for both gut and skin samples (Table 4).

Our second LMM analysis (with fish species, habitat and diet included as fixed factors and location as a random factor) of the effects on BC diversity (alpha (Chao1, PD) and beta (PCoA 1- 5) indices) showed that fish species, diets and habitats had significant effect on alpha diversity indices for gut samples but not for skin samples (Table 5). Moreover, fish species, diets and habitats had significant effect for both gut and skin samples for the beta diversity indices. Despite including diet and habitat in our model, fish species remained as a significant effect, indicating the species effect is likely more complex than simple species-specific diet or habitat preferences.

Table 3: PERMANOVA results for the effects of sample location, fish species and their interaction on bacterial community beta diversity (Bray-Curtis dissimilarity matrix) for both skin and gut samples. Only fish species captured at two or more locations were included in this analysis.

Variables	df	SS	R <sup>2</sup>	F value
<b>Gut</b>				
Location	2	10.5	0.13	17***
Fish species	9	6.6	0.08	2.4***
Fish species × location	10	6.3	0.07	2***
Residuals	181	55.7	0.70	
Total	202	79.2	1.00	
<b>Skin</b>				
Location	2	15.2	0.17	26***
Fish species	9	5.9	0.06	2.2***
Fish species × location	10	6.3	0.07	2.1***
Residuals	202	59	0.68	
Total	223	86.6	1.00	

Significance codes:  $P \leq 0.001$ \*\*\*

Table 4. Summary of the LMM outcomes testing for the effects of host fish species, sample location and their interaction on alpha diversity (Chao1, PD) and beta diversity (PCoA 1-5 from the Bray-Curtis dissimilarity matrices) of the gut and skin microbiome bacterial communities across 17 species of fish and three capture locations in the Great Lakes.

<b>Gut</b>			<b>Skin</b>		
<b>Chao1</b>			<b>Chao1</b>		
<b>Variables</b>	<b>df</b>	<b>F value</b>	<b>Variables</b>	<b>df</b>	<b>F value</b>
Location	2	1.4	Location	2	46.8***
Fish species	16	2.7***a	Fish species	16	1.3
Location ´ fish species	9	1.5	Location ´ fish species	9	3.9***
<b>PD</b>			<b>PD</b>		
Location	2	1.5	Location	2	55***
Fish species	16	2.85***	Fish species	16	1.8*
Location ´ fish species	9	1.7	Location ´ fish species	9	2.9**
<b>PCoA axis1</b>			<b>PCoA axis1</b>		
Location	2	92***	Location	2	215***
Fish species	16	1.8*	Fish species	16	3.7***
Location ´ fish species	9	2.4**	Location ´ fish species	9	2.1*
<b>PCoA axis2</b>			<b>PCoA axis2</b>		
Location	2	23.5***	Location	2	32.8***
Fish species	16	4.9***	Fish species	16	5.2***
Location ´ fish species	9	3.3***	Location ´ fish species	9	4.3***
<b>PCoA axis3</b>			<b>PCoA axis3</b>		
Location	2	26.8***	Location	2	49***
Fish species	16	5***	Fish species	16	4***
Location ´ fish species	9	5***	Location ´ fish species	9	6.2***
<b>PCoA axis4</b>			<b>PCoA axis4</b>		
Location	2	3.3*	Location	2	18.7***
Fish species	16	8.6***	Fish species	16	12.7***
Location ´ fish species	9	1.2	Location ´ fish species	9	4.7***
<b>PCoA axis5</b>			<b>PCoA axis5</b>		
Location	2	17.5***	Location	2	2.6
Fish species	16	2.3**	Fish species	16	4.7***
Location ´ fish species	9	2.5**	Location ´ fish species	9	4.9***

a. Significance codes: 0.01 < P ≤ 0.05\*, 0.001 < P ≤ 0.01\*\*, P ≤ 0.001\*\*\*

Table 5. Summary of the LMM outcomes testing for the effects of host fish species, habitats, and diets on alpha diversity (Chao1, PD) and beta diversity (PCoA 1-5) of the gut and skin microbiome bacterial communities across 17 species of fish and three habitats of the Great Lakes.

Gut			Skin		
Chao1			Chao1		
Variables	df	F value	Variables	df	F value
Habitat	2	4.4 <sup>***a</sup>	Habitat	2	1
Diet	7	6.3 <sup>***</sup>	Diet	7	1
Fish species	8	2.4 <sup>*</sup>	Fish species	7	1.6
PD			PD		
Habitat	2	2.8	Habitat	2	0.3
Diet	7	3.1 <sup>**</sup>	Diet	7	0.4
Fish species	8	3.2 <sup>**</sup>	Fish species	7	0.2
PCoA axis1			PCoA axis1		
Habitat	2	1.5	Habitat	2	4.9 <sup>**</sup>
Diet	7	1	Diet	7	5.6 <sup>***</sup>
Fish species	8	4.5 <sup>***</sup>	Fish species	7	2.2 <sup>*</sup>
PCoA axis2			PCoA axis2		
Habitat	2	0.6	Habitat	2	6.4 <sup>**</sup>
Diet	7	3.4 <sup>**</sup>	Diet	7	5.2 <sup>***</sup>
Fish species	8	2.7 <sup>**</sup>	Fish species	7	3.5 <sup>**</sup>
PCoA axis3			PCoA axis3		
Habitat	2	11.8 <sup>***</sup>	Habitat	2	7.5 <sup>***</sup>
Diet	7	7.8 <sup>***</sup>	Diet	7	6.4 <sup>***</sup>
Fish species	8	2.4 <sup>*</sup>	Fish species	7	2.2 <sup>*</sup>
PCoA axis4			PCoA axis4		
Habitat	2	9 <sup>***</sup>	Habitat	2	42.7 <sup>***</sup>
Diet	7	16 <sup>***</sup>	Diet	7	11 <sup>***</sup>
Fish species	8	4.6 <sup>***</sup>	Fish species	7	14.2 <sup>***</sup>
PCoA axis5			PCoA axis5		
Habitat	2	1.7	Habitat	2	1
Diet	7	5 <sup>***</sup>	Diet	7	5.9 <sup>***</sup>
Fish species	8	1.1	Fish species	7	2.8 <sup>**</sup>

a. Significance codes: 0.01 < P ≤ 0.05\*, 0.001 < P ≤ 0.01\*\*, P ≤ 0.001\*\*\*

### Host-microbiome BC Phylosymbiosis

Mantel tests of pairwise correlations between host phylogenetic distances and pairwise Bray Curtis BC dissimilarity (across all locations) values revealed a significant, but weak, positive relationship between BC dissimilarity and host evolutionary distance for both gut (r= 0.18, P < 0.05) skin samples (r= 0.26, P < 0.01) supporting the phylosymbiosis for fish gut and skin samples. (Fig. 5).

Figure 5. Scatterplot of pairwise host phylogenetic distance vs pairwise Bray Curtis dissimilarity for both gut (a) and skin (b) samples. Samples were combined within host species. Host phylogenetic distance was estimated using of CO1 and CytB mitochondrial gene

sequences.

## Discussion

Diverse exogenous and endogenous factors have been reported to contribute to determining the composition of fish microbiomes (5, 14, 29). However, the relative contributions of those factors in determining the composition of the teleost fish microbiome remains poorly characterized (7, 29). Our study evaluated how the bacterial component of the gut and skin microbiomes in 17 wild freshwater fish species sampled at three locations is shaped according to the surrounding water, trophic guild, diet, host species, and host phylogeny. While the fish BCs (skin and gut) were different from the surrounding water BCs, sample location (Detroit River, Lake Erie, Lake Ontario) had a strong effect on the composition of both the gut and skin BCs across all species of fish. Moreover, host species as well as host species-by-location interactions were significant but did not explain as much variation in the fish BC composition as the location main effect. Importantly, when we included fish diet and habitat preferences in our analysis, diet and habitat were important, but host species was still significant – indicating that the fish species effect was phylogenetically consistent and independent of diet and habitat, consistent with long-term co-evolutionary effects. When we tested the relationship between host phylogenetic distance and BC dissimilarity, we found a significant correlation, consistent with phyllosymbiosis. Overall, our results indicate that the host's environment has a greater role than host-specific selection in the assembly and composition of both host-associated BCs (gut and skin) and must be accounted for in any assessment of host-specific effects on the microbiome; however, evolutionary relationships between the host and its associated microbiomes are also important contributors.

The aquatic microbial community is thought to be the main source for the bacterial component of fish microbiomes, for both the gut and skin (55). Nevertheless, even with the on-going and constant exposure to the surrounding aquatic BCs, studies indicate that fish harbor microbiomes that are distinct from the water microbiome (56, 57). Our work supports those previous findings; we showed that the BCs in the fish gut and skin microbiomes were highly distinct from the surrounding water BC. Similar to other studies, we found Proteobacteria, Actinobacteriota, and Bacteroidota were the most abundant phyla in the aquatic environment (28, 29, 58). Other studies report that Proteobacteria, Fusobacteria, Firmicutes, and Bacteroidetes often comprise up to 95% of the fish bacterial microbiota (6, 29, 58, 59), also consistent with our work. These divergent patterns of bacterial abundance is expected, as Proteobacteria play an important role in the growth of fishes through nutrient cycling and the mineralization of organic compounds (60), while Firmicutes and Fusobacteria have roles in fatty acid absorption, lipid metabolism, fermentative process, and degradation of oligosaccharides in fish (27). While, in our study, the dominant bacterial phyla in the fish microbiome had some overlap with the water BC, major differences were apparent, likely due to differences in the functional roles of the fish-associated versus aquatic microbiomes. While differences between the fish microbiomes and the surrounding water microbiome are expected, we were surprised by the high level of divergence of the fish skin BC – clearly the Teleost skin mucous BC plays important functional roles in the host's performance and does not simply reflect the surrounding water's BC.

While broad comparisons at the phylum level are valuable, a more detailed differential abundance analysis at the family level provides more specific insights into BC variation between gut and skin microbiomes. Our differential abundance analysis showed fundamental differences between the BCs of skin and gut across a diverse array of host fish species. For example, *Deinococcaceae* and *Exiguobacteraceae* were generally more abundant in the skin mucus BC, relative to the gut content BC. On the other hand, members of *Microbacteriaceae* and *Lachnospiraceae* were more common in the gut BC. Bacteria in the family *Deinococcaceae* are obligate aerobes, and have a high resistance to ionizing radiation (gamma- and/or ultra-violet (UV) radiation) (61). This makes it unsurprising that *Deinococcaceae* was more abundant in the skin microbiome, as skin experiences more exposure to solar radiation than the gut. Their resistance to ionizing radiation may also contribute to *Deinococcaceae* being reported in habitats associated with high levels of solar radiation, including fish skin (62), hot springs (63) and rivers (64). In our study, we found a general pattern of elevated levels of *Deinococcaceae* in pelagic species (such as yellow perch, walleye) while lower levels were observed in deep water or benthic species (such as brown trout, lake trout, and round goby). The prevalence of *Exiguobacterium* in the skin BC in this study is consistent with its reported occurrence under a large range of environmental conditions (65), including fresh water (66) and skin in humans (67). So perhaps one reason for the elevated abundance of *Exiguobacterium* in fish skin samples relative to the gut samples in our study is the ability of *Exiguobacterium* to thrive under highly variable conditions (68), an important consideration for fish skin microbiomes that are exposed to considerable environmental variation relative to the gut habitat. Our detection of *Microbacteriaceae* at higher levels in the gut BC is consistent with previous work that reported them in various terrestrial and aquatic ecosystems (69), as well as associated with fish at various life stages (70-72). The association of *Lachnospiraceae* with the fish gut BC likely reflects their important functional role as intestinal symbionts of vertebrates (73), acting as butyrate producers in the intestinal microbiome. Members of *Lachnospiraceae* have been previously reported in the fish gut (74, 75), and indeed have been shown to have a symbiotic link to their host in surgeonfish (73).

Finally, we found substantially elevated levels of *Enterobacteriaceae* in the fish skin and gut samples, relative to the water samples (Fig. S1). Dominance of *Enterobacteriaceae* taxa in fish microbiomes has been widely reported in freshwater fish microbiomes (76-78) likely reflecting the importance of these bacteria for fish health and homeostasis (28, 79).

A large volume of published work shows that both endogenous and exogenous factors contribute to the Teleost microbiome composition (4, 5, 7, 8). Endogenous factors can act at the individual level to drive variation in the microbiome (e.g., via life history, host health status or host gene expression), as well as at the population level (e.g., via adaptation to local selection pressures) or even the species-level (e.g., via genomic variation and phylogenetic ancestry) (80). On the other hand, exogenous abiotic (climate, water chemistry, geography, etc.) and biotic (such as water microbiome) factors can contribute to variation in microbiome composition as well (81). Our analyses of alpha and beta diversity indices for both the skin and gut BCs showed significant effects of location, habitat (exogenous), diet and host fish species (endogenous). A variety of studies have reported both environmental and host species effects on the gut and skin microbiome composition in fishes (5, 29, 82, 83). Generally, the skin microbiota is reported to be more affected by environmental factors than the gut microbiome (8, 29), which is not surprising, given the close contact between the aquatic environment and fish skin habitat (84). Surrounding environments such as water and sediment are thought to be major sources of skin and gut microbiome bacteria (85, 86). In this study, sample location dominated host species effects, with, as expected, a stronger effect for the skin BC ( $R^2 = 0.17$ ) than for the gut BC ( $R^2 = 0.13$ ); although host species effects were still substantial (skin BC ( $R^2 = 0.05$ ); gut BC ( $R^2 = 0.05$ ). This pattern of effects on skin versus gut BCs was despite the strong BC divergence between both fish BCs and the water microbiomes. However, our Kruskal-Wallis also showed that skin BCs were more similar to that found in the water than the gut BCs. As the skin is in constant direct contact with the surrounding water microbiome, the skin BCs would be expected to reflect at least part of the bacteriological composition of the surrounding water (87). By contrast, the gut microbiome is known to be strongly influenced by host-related factors and diet (88). In this study we included 17 different fish species sampled at three different locations, and while we found a strong host species and location effects for both gut and skin, we recognized that those relationships may actually reflect local dietary/habitat preference variation among the study species (83, 89). Thus, our reported exogenous factor (location), may actually include a component of endogenous effects. This is further supported by the significant species-by-location interaction effect; such an effect reflects species-specific sample location effects, which would logically be due to differences in host diet, at least for the gut microbiome BC. Moreover, our study showed approximately equal effects of host species effects on the gut and skin BCs, perhaps due to the inclusion of multiple host species that utilized the aquatic habitat quite differently. Our observed host fish species effects on both gut and skin microbiome BCs agree with previous research showing interindividual, population, and species variation for microbial community composition (90), all of which were interpreted as due to host endogenous factors. Given that the gut microbiome habitat is highly controlled by the host's physiology, only bacterial species adapted to that environment would be expected to thrive in the gut, hence the host should have considerable effect on the gut BC composition (29). The specific mechanisms driving variation in the fish BC are still unclear, despite substantial research (including this work), likely due to complex interactions among possible mechanisms.

Microbiome similarity among species in a community is predicted to decrease with increasing evolutionary divergence of the host organisms (17), this is the basis for phylosymbiosis. However, phylosymbiotic patterns can result from diverse factors, including phenotypic divergence among fishes that are phylogenetically distant (15), co-evolution between the individual bacteria in the microbiome and the host (91) and even patterns of host behaviour or life history that may be correlated with phylogeny but also indirectly affect the microbiome (e.g., feeding preferences) (22). Additionally, evolutionary processes such as selection and drift can also shape the species relatedness and their associated BC, thus resulting in phylosymbiosis (16, 92). Numerous studies have documented an effect of the host fish species on microbiome BCs (4, 79, 82). We showed correlations between BC composition divergence and host fish taxonomic divergence for both the gut and skin microbiome BCs. While species-specific diet differences do contribute to BC similarity; the species effects were still present after correcting for diet, indicating that the mechanism behind our observed phylosymbiosis cannot be due to diet alone. Previous studies showed that host-specific microbiomes are a widespread pattern in nature, occurring in many host organisms (16), including mammals (19), birds (93), insects (94), and fish (4, 95). Although some bacterial lineages may still co-diversify with hosts, it is important to note that phylosymbiosis by itself is not an indicator of host-microbiome adaptive co-evolution. Evidence for phylosymbiosis in non-mammalian vertebrate animals is incomplete and inconsistent (16). For example, some studies in fish showed evidence for phylosymbiosis (4), whereas others report mixed or weak evidence (7, 29). Curiously, despite the fact that multiple studies (including ours) have shown that the fish skin microbiome is generally more affected by the environment than the gut microbiome (29, 56) we found a stronger signal of phylosymbiosis for the fish skin BC than for the gut BC. This was not expected, as the fish skin microhabitat is much more affected by environmental physicochemical parameters (29), not to mention the water microbiome. Clearly, the host species still has a substantial effect on even their peripheral microbiomes, highlighting the functional importance of all host-associated microbiomes. Given that our sampled fishes included 17 species representing 7 orders, our phylosymbiosis analysis is powerful and provides a starting point for future evaluations of the role of co-evolution between host organisms and their microbiome communities.

Overall, our findings contribute to the characterization of the modulators of microbiome composition and diversity across fish taxa. While many studies have characterized fish microbiome BCs, yet few of those studies included multiple species sampled in the wild across multiple locations. Our study design provided a robust test of the relative effects of habitat, host diet and host species on the BCs of two key microbiomes associated with fish health and fitness. Not surprisingly, we found that the fish microbiome BCs are distinct from the aquatic environmental BC, but that sampling location had a strong effect on BC composition, nevertheless. Curiously, we found strong host species-by-location interaction effects for both skin and gut microbiome BCs, indicating that the species effects varied among the three sampled locations, possibly due to local fish diet and/or habitat-use differences. As expected, we also found a significant, but less strong effect of host fish species on both the gut and skin microbiome BCs. Based on the host fish species effect that persisted after correcting for diet and habitat preferences, we tested for, and identified, significant phylosymbiotic signals between host phylogeny and both the gut and skin microbiome BCs. This suggests that both the gut and skin BCs co-evolved with their host species, although ecological covariation also contributes substantially since the variance in BC similarity explained was modest. Investigations of the nature of fish-microbe associations, and whether they are sustained, functional relationships or transient effects of fish and habitat associations are critical to further our understanding of the potential beneficial interactions between hosts and their microbiomes.

## **Declarations**

### **Ethics approval and Consent to participate**

All fish handling was performed by other agencies; however, we also had Canadian Council for Animal Care permitting for handling and humanely euthanizing fish.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The raw 16S rRNA gene sequencing data are available at the Sequence Read Archive of NCBI with PRJNA701818 BioProject accession number.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

J.S, S.R.C, D.D.H. conceived and planned the experiments. J.S carried out field work. J.S. carried out the wet laboratory sample preparations and experiments. All authors contributed to selecting the models and the computational framework for the data analysis. J.S., S.R.C, T.B.J and D.D.H. contributed to the interpretation of the results. J.S., took the lead in performing the research, analyzing data and writing the manuscript. S.R.C, T.B.J, and D.D.H., provided critical feedback and helped shape the research, analysis and manuscript.

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## Figures

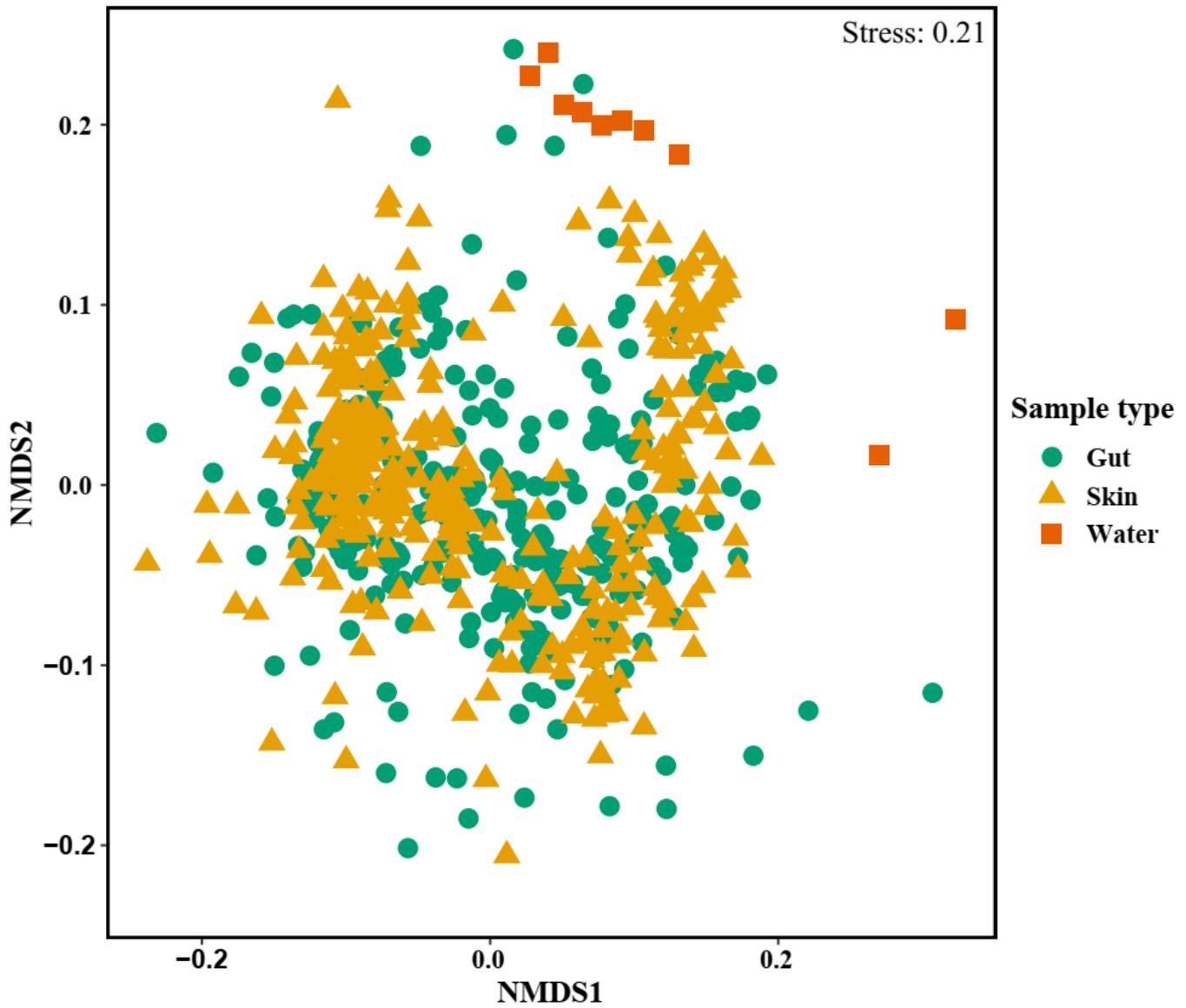


Figure 1

NMDS plot of BCs associated with fish gut, skin, and water samples based on Bray-Curtis dissimilarity matrix. Shapes and colours are based on sample types.



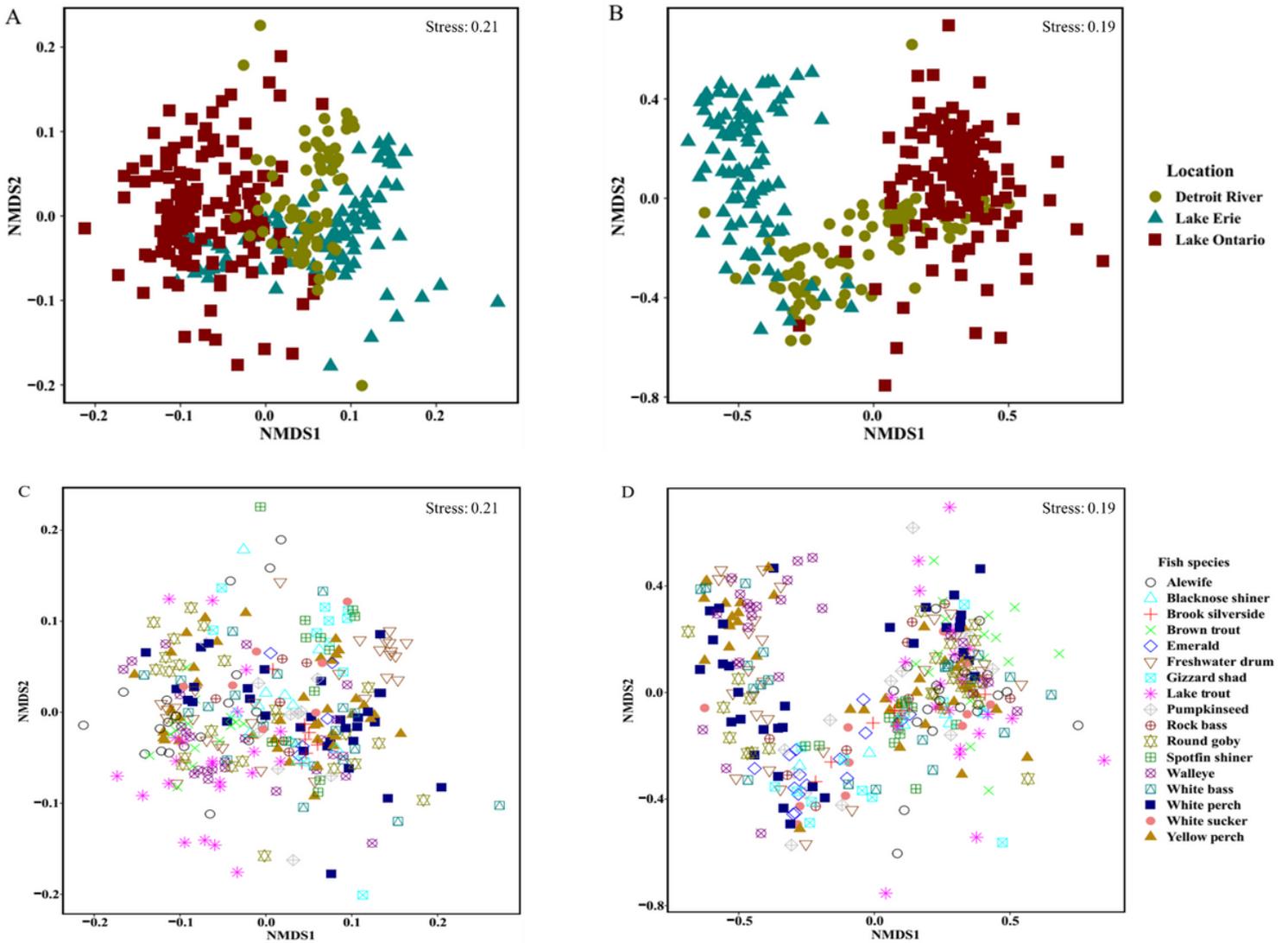
Figure 2

Stacked bar plots showing relative abundance of gut bacterial community composition presented at the family level for all 17 fish species at the three sample locations (DR; Detroit River, LE; Lake Erie, LO; Lake Ontario). Each bar is representative of an individual fish within that species.



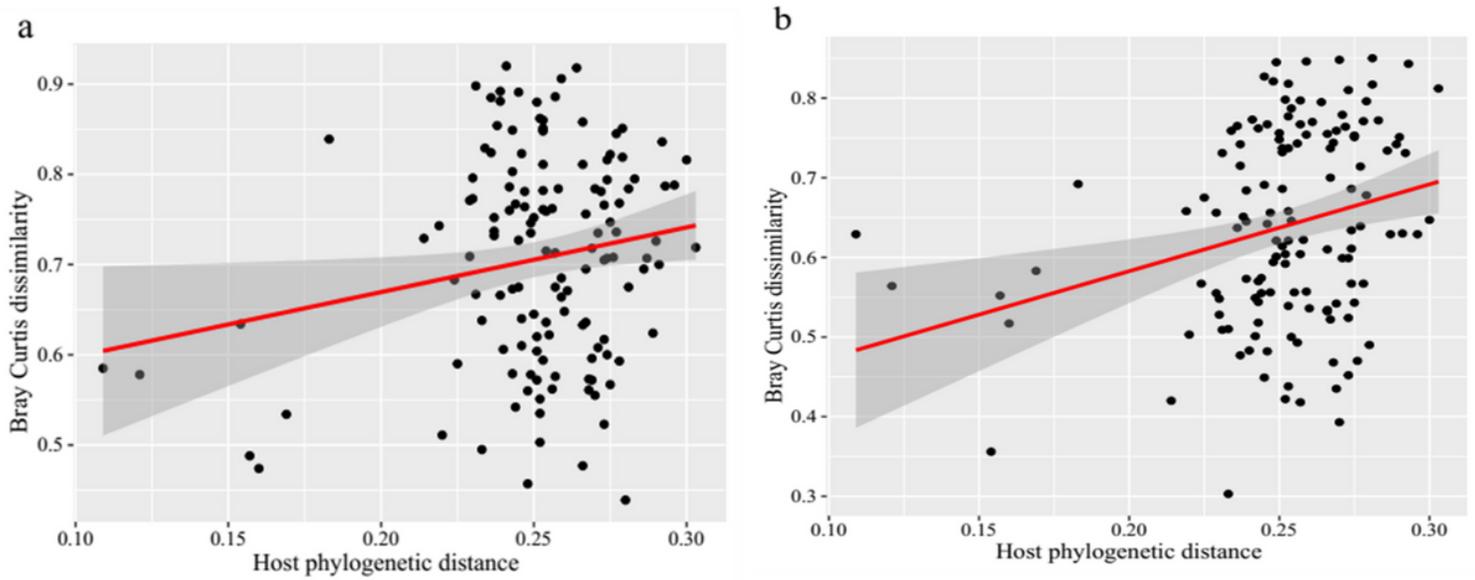
Figure 3

Stacked bar plots showing relative abundance of skin bacterial community composition presented at the family level for all 17 fish species at the three sample locations (DR; Detroit River, LE; Lake Erie, LO; Lake Ontario). Each bar is representative of an individual fish within that species.



**Figure 4**

NMDS scatter plots of gut and skin microbiome bacterial community composition based on Bray-Curtis distance matrices for 17 species of fish sampled at three locations (Detroit River, Lake Erie and Lake Ontario). The left panels A and C) show the NMDS for the gut microbiome bacterial community compositions, while the right panels (B, D) show the NMDS for the skin microbiome bacterial communities in the same fish. The NMDS plots A and B are coded to show the location of capture for each fish, the panels C and D are coded to show the species of each fish sampled.



**Figure 5**

Scatterplot of pairwise host phylogenetic distance vs pairwise Bray-Curtis dissimilarity for both gut (a) and skin (b) samples. Samples were combined within host species. Host phylogenetic distance was estimated using CO1 and CytB mitochondrial gene sequences.

## Supplementary Files

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

- [FishmicrobiomeSupplementV5.docx](#)