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Core microbiomes found in habitats with sandy bottom substratum are the most phylogenetically diverse among trophic level three ray-finned fish midgut samples

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SUMMARY Fish gut microbiomes play an indispensable role in regulating fish growth, behavior and immune health, yet little is known about the environmental factors that influence its composition. We investigated how habitat substrata affects the midgut microbiome in carnivorous ray-finned fish, hypothesizing that fish from similar substrata have similar midgut microbial compositions. Using alpha diversity metrics, midgut samples demonstrate the highest variety and abundance of microbial species compared to other body sampling sites. Midgut samples from fish found in habitats with kelp forest, sandy bottom and rocky reef substrata exhibit distinct core microbiomes, only sharing one common core species: *Pseudoalteromonas sp*. In addition, we characterized the relative and total abundance of midgut samples at a phylum level and identified Proteobacteria, followed by Bacteroidetes and Firmicutes, to be the most dominant phyla in the core midgut microbiota. The midgut microbiomes from fish living in habitats with sandy bottom substratum are characterized by the key indicator species *Synechococcus sp.* CC9902 and *Psychromonas sp.* Overall, this study demonstrates that the fish gut microbiome is associated with the fish's habitat, with the key species identified providing the foundation in understanding the major phyla associated with carnivorous fish microbiota. We hope to extend this knowledge into developing probiotics to promote fish digestive and immune health, as well as technology to monitor fish populations.

INTRODUCTION

A diverse fish gut microbiome is important in regulating fish digestive and immune health (1), yet the factors that influence fish gut microbiome development remain unclear. The most dominant phyla in fish gut microbiota are Proteobacteria, Firmicutes, and Bacteroidetes, though the proportion of these phyla varies with host taxonomy (2). Although the fish gut microbiome is influenced by species-specific factors such as host genetics and diet (3), the gut composition is predominantly modulated by factors related to host habitat: salinity, pH, temperature, light intensity, and diet composition (2, 4). However, the link between habitat associated factors and the impact on fish gut microbiomes is still poorly understood (5).

Within the context of fish microecology, substratum refers to the bottom floor on which an organism lives (6). Many species of fishes frequently interact with their substratum and require an appropriate substratum to exhibit important natural behaviors such as spawning (7). It is reasonable to assume that through these interactions, microorganisms living in the substratum can be potentially ingested, therefore affecting the gut microbiota. In addition, marine sediments have variations in oxygen and organic carbon availability that affect the

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habitability of aerobes and anaerobes (8). Hence, fish gut microbial composition can differ as a result of substratum composition.

The dataset used in this paper originates from a study done by Minich et al. in 2022, in which they analyzed the microbial composition of samples from the midgut, hindgut, gill, and skin of a variety of wild marine fishes from off the coast of Southern California and New York state (5). They analyzed the environmental and biological factors to identify patterns that influence fish microbial diversity. They found that the greatest predictor of microbial diversity in fishes is the body sampling site, in which host-associated microbial communities are significantly associated with host phylogenetic relationships in the gill, skin, and hindgut, but not midgut (5). Although various factors such as biomass, depth, and swimming behavior have been explored (5), no link has been established between the substrata and the fish gut microbiome. As midgut is least affected by host phylogeny (5), we decided to investigate how substrata affect the microbial diversity and composition of fish midgut microbiota.

In order to reduce major phylogenetic and physiological variations that could pose as compounding factors (9), the scope was limited to only include fishes that were of trophic level three and from the class Actinopterygii, commonly referred to as ray-finned fishes. The subsetting of trophic level three fish was done to mitigate differences in lifestyle as prior studies have indicated that there are distinct differences in gut microbial species across each trophic level (10). Subsetting of ray-finned fish was done to exclude fish with drastically different gut anatomy such as sharks, rays (class Chondrichthyes) and hagfishes (class Myxini). Sharks and rays have a spiral valve, which is a spiral-shaped section of the intestine that increases surface area, slows the passage of food, and prevents flow in the opposite direction (11). The effect that the spiral-valve has on the microbiome is currently unknown, but considering it affects gut mobility it could be a confounding variable. Hagfish do not have a spiral valve, however they fast for up to 11 months at a time, exerting extreme selective pressure on their gut microbiome (12). Narrowing the ken onto just trophic level three ray-finned fish focuses the investigation on fish with similar lifestyles and gut assembly.

With the knowledge that a plethora of factors such as nutrient availability (4), host genetics and diet (3) regulate the fish gut microbiome, our study focused on measuring the impact of the substrata on the midgut microbiome of trophic level three ray-finned fish. As previous studies have suggested the fish gut microbial community was more predominantly regulated by host habitat than by genetic factors (2), we hypothesized that substrata would be a driving factor of the gut microbiome, with samples from the same substratum sharing similarities in microbial composition. Here, we aimed to determine if midgut samples possessed higher microbial diversity than other body sites, to explore if those variations could be tied to differences in substrata, and to establish if samples from the same substratum carried similarities in the gut microbiome composition.

We found that the richest body site of trophic level three ray-finned fish is the midgut, with samples from each substrata having a distinct core microbiome, solely sharing *Pseudoalteromonas sp*. We also observed Proteobacteria, followed by Bacteroidetes and Firmicutes, to be the most dominant phyla in the core midgut microbiome. The sandy bottom substratum was the most even community, with *Synechococcus sp.* CC9902 and *Psychromonas sp.* being identified as indicator species for the substratum. These findings suggest that substrata is a mild driver of gut microbial diversity in trophic level three ray-finned fish, providing fundamental knowledge for understanding how gut microbes regulate fish health.

METHODS AND MATERIALS

Data acquisition. To investigate the assembly of marine fish mucosal microbiomes, Minich et al. analyzed microbiota from 101 species of fish from off the coasts of Southern California and New York state (5). The fish were wild caught using primarily hook and line, spear, or trawls (for deep sea fishes) (5). These authors were the only source using this dataset at their publication date. The dataset includes microbiota data collected from four separate fish mucosal locations: gill, skin, midgut, and hindgut. The sample metadata represents ten different substrata.

Prima facie data filtering in R. The manifest and metadata files were filtered to keep only samples from class Actinopterygii (ray-finned fishes) and trophic level three using the dplyr package in R (13). As some of the output statistics of QIIME 2 are reported from a random sample of the data (14), the data was filtered prior to working in QIIME2 such that sample statistics, for instance base quality, would be more representative of our population of interest.

Taxonomic and phylogenetic analysis through QIIME 2. The data was demultiplexed using q2-demux and then the sequences were denoised with Divisive Amplicon Denoising Algorithm 2 (DADA2) to regulate the quality of our sequences (15). Reads were truncated to a length of 235 nucleotides, as selected with visualization from QIIME 2 View, to excise the most concentrated region of poor base quality.

A naive bayes classifier (16, 17) was trained against the V4 region of 16S rRNA (18, 19) using the primers specified by Minich et al. (5) to increase the probability of a correct taxonomic assignment (20, 21). After completing taxonomic analysis using q2-feature-classifier, filtering was performed to remove features that were either mitochondrial, chloroplastic, or unassigned using feature-table. The key outputs of a feature table from q2-alignment, a taxonomy table, and a rooted phylogenetic tree from q2-phylogeny were then exported to R for downstream analyses (22, 23, 24).

Statistical analysis of all body sampling locations and substrata. A phyloseq object was created and rarefied to a sequencing depth of 4395 to maximize the number of observed features while preserving samples for downstream analyses (N=43). Alpha diversity using Chao1 (25) and Shannon (26) metrics and beta diversity using Weighted UniFrac (27) were calculated for body sampling sites and substrata with the phyloseq (28), ape (29), tidyverse (30), and vegan (31) packages. Pairwise comparisons within sampling sites and substrata was done using PERMANOVA (31). Uniform Manifold Approximation and Projection (UMAP) was used to cluster the distribution of ASVs to look for significant associates between metadata categories (32), using the tidyverse (30), umap (33), and Rcolorbrewer (34) packages in R.

Statistical analysis of only midgut samples from specific substrata. Following a preliminary investigation of all body sites, the data was subset to samples from the midgut. Only samples from kelp forest, rocky reef, or sandy bottom substrata were kept because of minimum sample number restrictions. The phyloseq object was filtered to include only midgut samples from the three specified substrata then used to calculate alpha diversity via Faith's PD (35), Chao1 (25), and Shannon (26) diversity metrics, and to create corresponding boxplots and significance analyses (Kruskal-Wallis Test (36) for Faith's PD and Shannon, PERMANOVA for Chao1). The Chao1 index was used to perform pairwise PERMANOVA analyses on each pair of substrata to pinpoint any significant substratum. Weighted UniFrac (27) beta diversity was used to create a PCoA plot, and the corresponding significance across substrata was calculated using PERMANOVA (31).

Core microbiome analysis and indicator species identification. To identify core microbiome species across midgut microbiota samples from the three substrata (N=43), a core microbiome analysis (9) was run using detection = 0.001 and prevalence = 0.5, and the tidyverse (30), phyloseq (28), microbiome (37) and ggVennDiagram (38) packages were used to visualize distinct and overlapping species in a venn diagram. To identify key microbes amongst substrata, we used the indicspecies package developed by Cáceres et al. (39) in R to calculate Dufrêne and Legendre indicator values (40). The complete midgut microbiome analysis workflow was illustrated in Figure 1.

RESULTS

Microbiota from midgut and sandy bottom substratum samples demonstrate the highest microbial alpha diversity for body site and substrata respectively. To begin our analysis, we explored microbial diversity across body sampling locations and substrata by performing a general alpha and beta diversity analysis on all trophic-three level fish and ray-finned fish. Chao1 and Shannon diversity metrics showed that the midgut has the most diverse

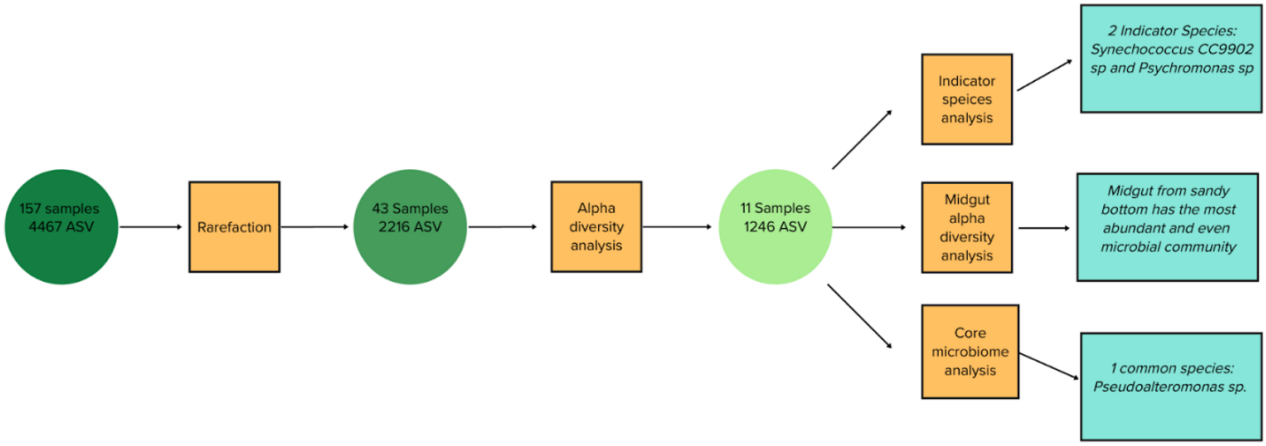

FIG. 1 Midgut microbiome analysis workflow. The midgut analysis workflow detailing the main data processing steps described in the method and the number of samples funneled into each step of the analysis.

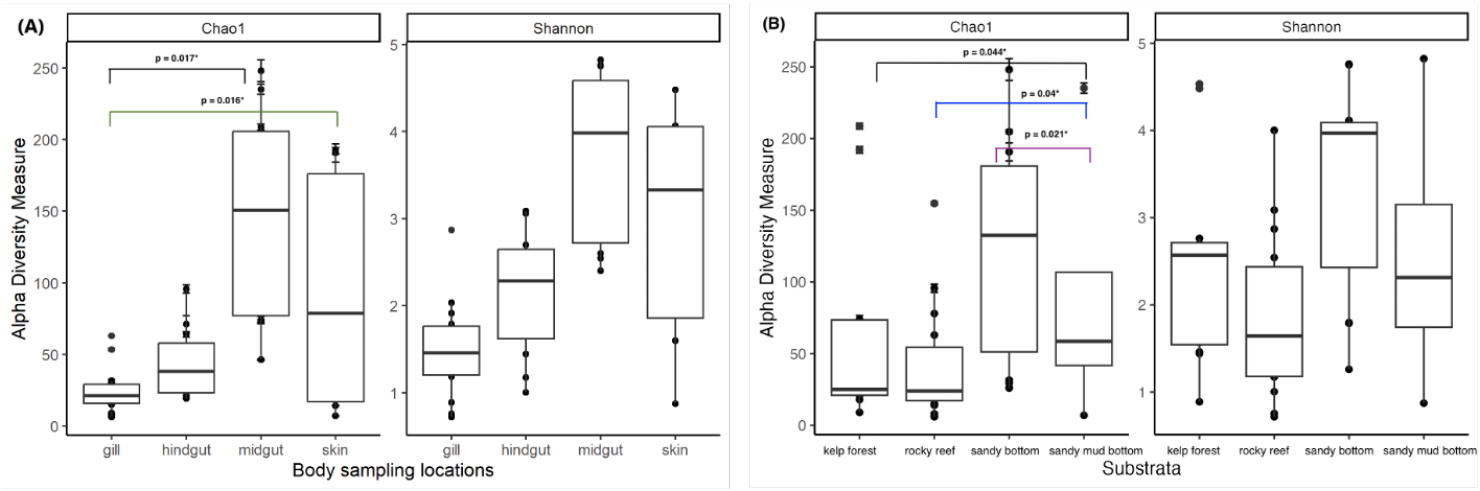

FIG. 2 Microbiomes in midgut samples and sandy bottom substratum demonstrate the highest alpha diversity for body site and substrata respectively. Alpha diversity using Chao1 (richness) and Shannon (richness and evenness) was run in N=43 for **(A)** body sampling locations and **(B)** substrata in Rstudio (version 2022.12.0+353). Significance in alpha diversity in Chao1 was assessed using PERMANOVA and only statistically significant pairwise comparisons were noted. **(A)** $p = 0.017$ * (black) for gill vs midgut, $p = 0.016$ * (green) for gill vs skin. **(B)** $p = 0.044$ * (black) for kelp forest vs sandy mud bottom, $p = 0.040$ * (blue) for rocky reef vs sandy mud bottom and $p = 0.021$ * (magenta) for sandy bottom vs sandy mud bottom.

community in terms of species richness and abundance, ahead of the skin, hindgut, and gill (Figure 2A). In addition, we found that samples from the sandy bottom substratum have the richest species, while samples from kelp forest, rocky reef and sandy mud bottom substrata have relatively lower richness, but greater evenness (Figure 2B).

Pairwise comparisons across groups revealed that the gill and skin samples have the most different microbial diversity, with gill and midgut samples having significantly different microbial communities as well (Figure 2A). In addition, sandy mud bottom samples have significantly different microbial communities compared to those from kelp forest, rocky reef and sandy bottom (Figure 2B). Because midgut samples have the highest number of species, we decided to investigate how substrata affects the microbial composition of fish midgut. The sandy mud bottom substratum possessed a single midgut sample and was therefore eliminated from further analyses. The rest of our analysis focused on the extent to which midgut samples from the kelp forest, rocky reef and sandy bottom substrata are similar to one another.

No substratum shows significantly different midgut microbial diversity from other substratum types. We used alpha diversity metrics to investigate how the midgut microbial communities differ across substrata (Figure 3). The results suggest no significant difference

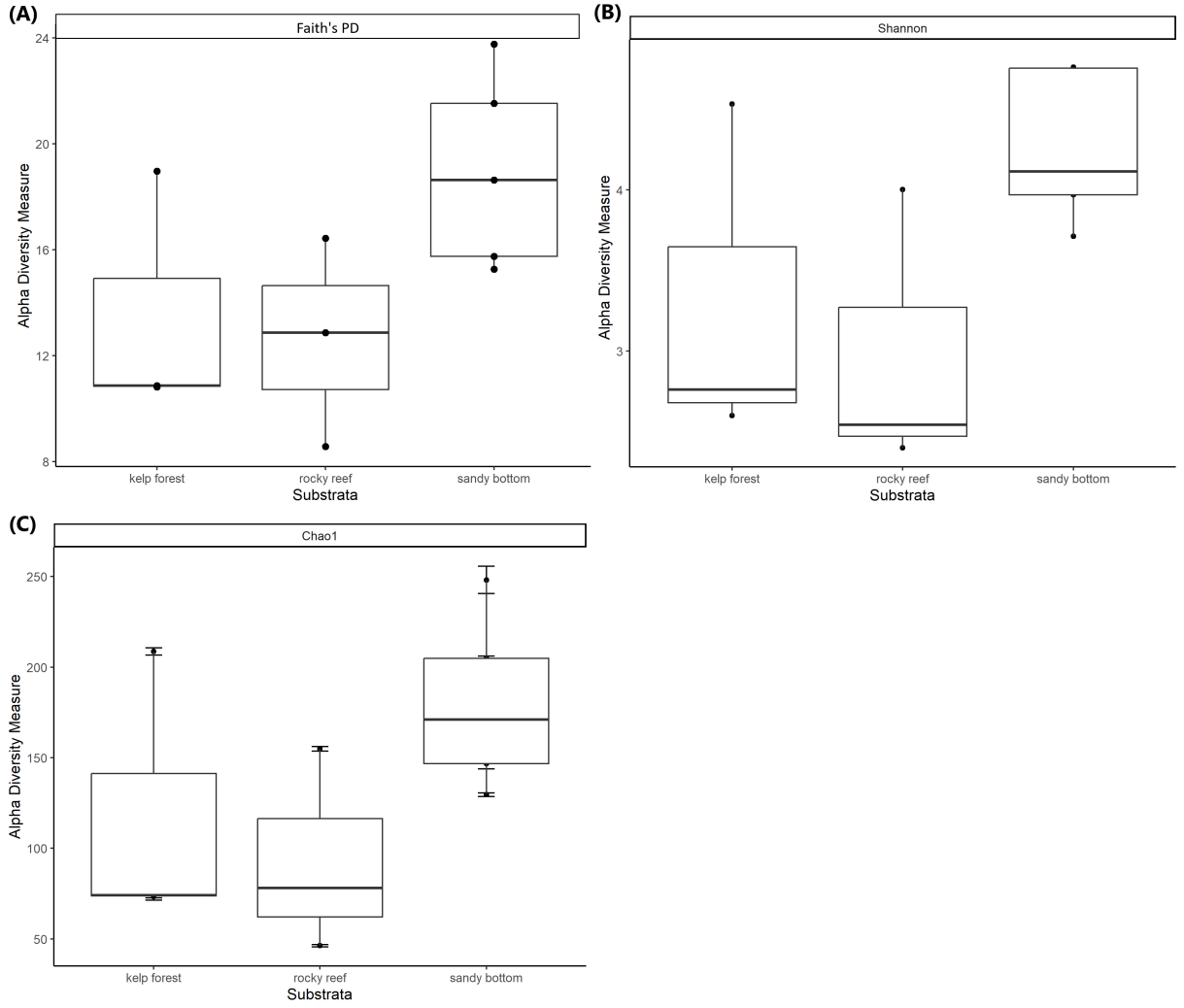

FIG. 3 There were no significant differences in midgut microbial abundance across substrata. N=3 for rocky reef, N=3 for kelp forest, and N=5 for sandy bottom. **(A)** Faith's phylogenetic diversity quantified microbial alpha diversity across substrata in midgut samples, error bars indicate mean ± SE, significance calculated via Kruskal-Wallis Test. **(B)** Shannon index significance box plot measures Shannon's microbial alpha diversity across substrata in midgut samples, error bars indicate mean ± SE, significance calculated via Kruskal-Wallis Test. **(C)** Chao1 index quantifies microbial alpha diversity across substrata in midgut samples, error bars indicate mean ± SE, significance calculated via PERMANOVA.

in species richness between the samples based on substrata. Subsequential pairwise Chao1 tests on the substrata also indicate that no pairing of two substrata possesses significant differences in species richness. To investigate the beta diversity of our midgut samples, we created a Weighted UniFrac distance PCoA plot (Figure S2). It again suggests that the samples from all three substrata do not significantly differ in the overall microbial composition.

Midgut of fish from rocky reef, sandy bottom and kelp forest substrata have distinct core microbiomes and share one common species: *Pseudoalteromonas sp.* To further investigate the extent to which different substrata impacts the microbial composition of the fish gut microbiome, we used a core microbiome analysis to identify the most prevalent species amongst our samples. The midgut core microbiome species have almost no overlap across substrata, with only *Pseudoalteromonas sp.* being present in all midgut samples. Midgut samples from the rocky reef substratum have 16 unique core species, while midgut samples from sandy bottom and kelp forest substrata each have ten unique species (Figure 4).

This highlights the diversity in microbial species across habitats and suggests that the diversity in the aquatic microbial community can potentially contribute to the fish gut microbiome.

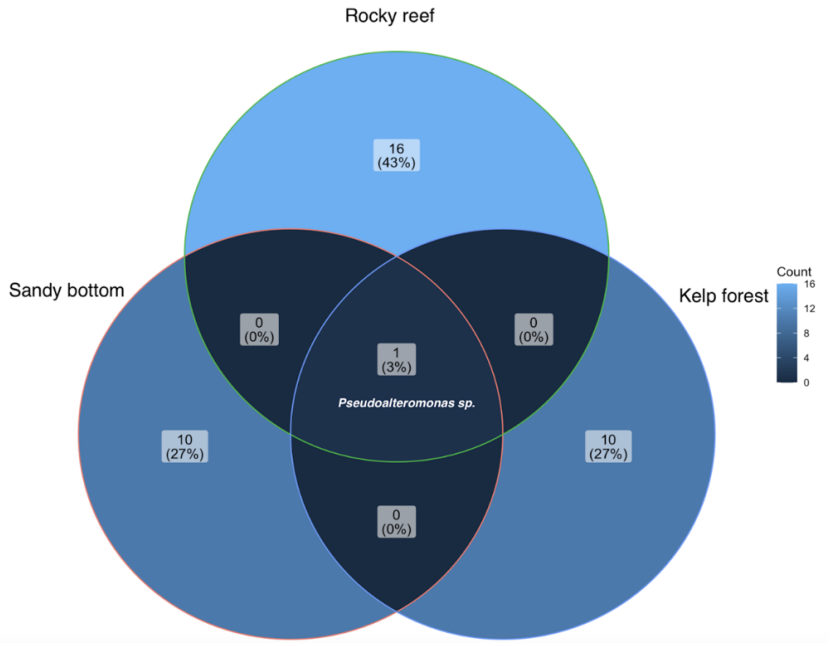

FIG. 4 Midgut of fish from rocky reef, sandy bottom and kelp forest substrata have distinct core microbiomes but share a common species. Core microbiome species found in the midgut of fish from rocky reef, sandy bottom and kelp forest substrata with N=43 were visualized in a three-way Venn diagram created by the core microbiome analysis in Rstudio (version 2022.12.0+353). Using detection = 0.001 and prevalence = 0.5, we found 16 distinct midgut species in samples from the rocky reef substratum, ten distinct species from the sandy bottom substratum, and ten distinct species from the kelp forest substratum. The midgut of fish from these three habitats shares one common species: *Pseudoalteromonas sp*.

Midgut of fish from sandy bottom has the most even core microbiome; Proteobacteria and Bacteroidetes dominate the core microbiome in midgut samples across three substrata. To further identify the species that made up the midgut microbiomes from kelp forest, sandy bottom, and rocky reef substrata, bacterial phyla were retrieved from their corresponding ASVs. We identified eight bacterial phyla that populated the midgut microbiomes from three substrata to various degrees (Figure 5). Midgut microbiomes from the rocky reef substratum have the highest number of species (two species were Teleosts, an infra-class of Actinopterygii, and excluded from total abundance in midgut samples from the rocky reef substratum). Out of 15 bacterial species identified in rocky reef midgut samples, six were Bacteroidetes, six were Proteobacteria, two were Firmicutes and one was Verrucomicrobiota. Midgut from kelp forest and sandy bottom substrata each have ten core species, however the distribution of these species was drastically different. Midgut from the kelp forest substratum has ten core species from four distinct phyla: eight Proteobacteria, one Bacteroidetes, one Verrucomicrobiota and one Fusobacteriota. Midgut samples from the sandy bottom substrata also have ten core species, but from six different phyla: five Proteobacteria, two Bacteroidetes and one count each for Verrucomicrobiota, Cyanobacteria, Planctomycetota and Myxococcota. This reveals that although midgut samples from the rocky reef substratum have been identified to have the highest number of core species, midgut from the sandy bottom substratum has the highest variety of species that are most evenly distributed. Additionally, the most dominant phylum in midgut samples from kelp forest and sandy bottom substrata is Proteobacteria, whereas Bacteroidetes and Proteobacteria appeared to co-dominate in midgut samples from the rocky reef substratum.

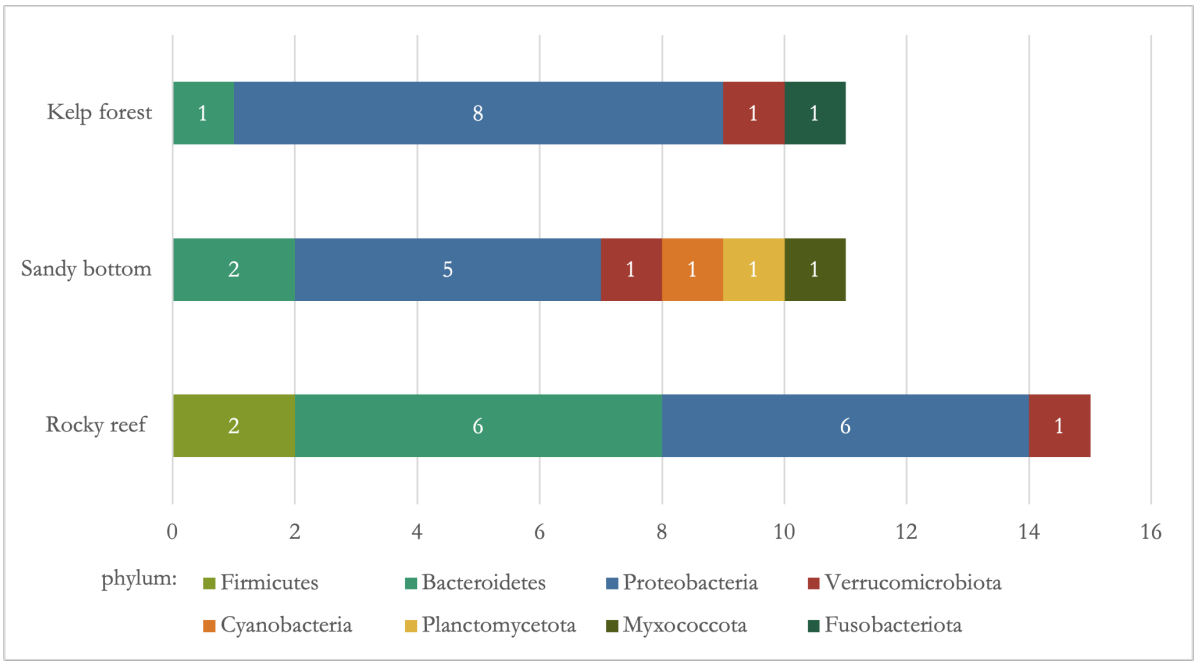

FIG. 5 Total abundance of midgut core microbiome phyla across substrata. Stacked bar chart of total abundance of midgut core microbiome phyla from kelp forest, sandy bottom and rocky reef substrata. Computed using Microsoft Excel for Mac (Version 16.72).

Two indicator species present for the sandy bottom substratum. We examined the microbial communities from midgut samples across substrata to determine whether or not certain bacterial species were significantly correlated to a particular habitat. We used the indicator value (INDVAL) method to comb our samples for indicator species in the sandy bottom, rocky bottom, and kelp forest substrata (39). Bacterial species marked as midgut indicator species for a specific substratum are displayed in Table 1.

We only found two indicator species, both for the sandy bottom substratum. These belonged to the genera *Synechococcus* and *Psychromonas*. Both indicator species we identified were congruent with members of our sandy bottom core microbiome, with *Psychromonas* belonging to the most abundant phylum in Proteobacteria and with *Synechococcus* being the only Cyanobacterium identified in either the core microbiome or indicator species analyses.

TABLE. 1 Midgut indicator species significantly ($p < 0.05$) associated with substrata as identified by indicator value (INDVAL) analysis.

Phylum	Class	Family	Indicator species	Substrata	INDVAL (*p*-value)
Cyanobacteria	Cyanophyceae	Synechococcales	*Synechococcus sp.* CC9902	Sandy bottom	0.894 (0.02)
Proteobacteria	Gammaproteobacteria	Alteromonadales	*Psychromonas sp.*	Sandy bottom	0.894 (0.03)

DISCUSSION

Fish gut microbiomes play a vital role in fish growth, behavior and immune health (41). Here, we explored the gut microbiomes of various body sampling sites of trophic level three ray-finned fish by environment. Using Chao1 and Shannon alpha diversity, we affirmed that the midgut microbiome has the highest diversity in terms of species richness and evenness. Previous studies have shown that Proteobacteria, Firmicutes, and Bacteroidetes represent 90% of the fish gut microbial community and that the host habitat is a greater determinator

of the microbiome composition than genetics (2, 42). As such, we hypothesized that substrata microbial composition might modulate the fish gut microbiome.

From our analysis by midgut (Figure 3), we found that midgut microbiomes from all three substrata did not possess significant differences in microbial abundance. Due to our small number of samples after filtering, there may be inaccuracies when running diversity metrics as statistical tests often lose power at small sample numbers, increasing the prevalence of false negatives (43). From our core microbiome analysis, we found that the core midgut communities from the three substrata are vastly different, each having distinct core microbiome species while only sharing *Pseudoalteromonas sp.* Additionally, we identified Proteobacteria, followed by Bacteroidetes, to be prevalent phyla across all midgut core microbiomes. Midgut samples from the sandy bottom substratum have the most phyla-even core microbiome, with two indicator species characterized in *Synechococcus sp.* CC9902 and *Psychromonas sp.*; both microbes having largely unknown roles within the microbiota of fish.

Through our core microbiome analysis, we identified a common species shared by all midgut samples from our three substrata. *Pseudoalteromonas* is a genus of marine bacteria commonly found in aquatic environments (44, 45). It is frequently associated with eukaryotic hosts in marine environments where it produces a range of biologically active extracellular compounds, including pathogen-protective antimicrobial compounds as well as proteases that are important to host metabolism (44). Forming biofilms in the fish gut environment, it aids food digestion and prevents colonization of pathogenic bacteria (46). It also produces alginate lyases that degrade alginate polysaccharide which provides the main energy source in algae-eating fish (46, 47, 48). In addition, the species *Pseudoalteromonas ruthenica* possesses antibacterial activity against the pathogenic bacteria *Edwardsiella piscicida*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*, being used a probiotic treatment to improve the gut health of fish in aquacultures (49, 50).

We then further investigated the differences in the composition of the core midgut microbiota with respect to habitat. Indeed, a phylum-level difference across three substrata was detected (Figure 5). The most prevalent phyla in our midgut samples are Proteobacteria and Bacteroidetes. Consistent with the literature, these phyla comprise a larger proportion of the gut microbiota (51), constituting 81.08% of all our samples in conjunction with Firmicutes. In mammals, a bloom of Proteobacteria is considered a sign of dysbiosis or instability in the gut microbial community, as many commensal Proteobacteria are opportunistic pathogens, infecting the host under specific conditions and facilitating inflammation (52, 53, 54). In fish however, Proteobacteria dominate the gut microbiota (55). Although the function of Proteobacteria in fish guts is unclear, its prevalence is largely due to the digestive system of fish, which unlike that of mammals, is unsegmented, allowing for ease of attachment (56). Other core microbiome phyla Bacteroides and Firmicutes have been established as the main material-metabolizing bacteria in the fish gut (57), in which Bacteroidetes promote carbohydrate metabolism while Firmicutes aid in energy harvesting (58).

Of our two microbes identified through INDVAL analysis, *Synechococcus sp.* CC9902 was the only identified Cyanobacteria (59) across all substrata. It has been previously characterized as a component of bacterioplankton (60) and is not known to play a role within the microbiota of fish. Bottom floor *Synechococcus sp.* CC9902 populations are known to negatively correlate with salinity and nitrite abundance, factors that fluctuate seasonally (61). Furthermore, *Synechococcus sp.* CC9902 serves as bacterial feedstock for planktonic protists, where its morphology and behavior are not associated with a reduction in grazing rates from other genera of *Synechococcus* (62). These factors make its presence as a core microbe and indicator species particularly puzzling. Without negative water column controls, we cannot dismiss the notion that *Synechococcus sp.* CC9902 abundance in the midgut is due to an overall abundance in the sandy bottom environment as opposed to any sort of biological significance.

On the other hand, *Psychromonas* has been documented as a potential member of the gut microbiome. *Psychromonas*, like its phylum Proteobacteria, is a highly diverse genus; known for being cold-tolerant, its members have been reported as free-living (63), possessing biofilm (64) and anti-biofilm activities (65), and residing in arctic (66), antarctic (67), and deep ocean (68) environments. One isolate in particular, *Psychromonas CDP1*, as dubbed by Zhang et

al., was found in the gut of amphipod *Hirondellea gigas* and noted to have a markedly reduced genome in comparison to free-living species (68). Zhang et al. tracked these changes to pathways believed to be essential for free-living *Psychromonas*, postulating that the bacterium's symbiotic relationship with its host allowed it to forgo some of its genes. As with *Synechococcus sp.* CC9902, the observed *Psychromonas* may simply just be abundant in the environment. However, without temperature data, we cannot conclude if these sandy bottom samples were taken in a region conducive to supporting the presence of *Psychromonas*. Again there is clear doubt upon the validity of the *Psychromonas* identification as well as its potential biological significance if any.

These findings prompt the questions of whether these core microbiome species outcompete their environmental bacterial taxa in the aquatic habitat or whether they have been selected in the midgut by the host itself (69, 70). The answer is not simple. An interplay of habitat-specific factors and host-specific factors determines the constitution of the fish gut microbiome. Some studies suggest that salinity, light intensity and water temperature influence aquatic microbial communities, altering fish diet and their gut microbiota (71, 72). Strong evidence of host genetics, developmental stage, immune status and other host specific pressures on the gut microbiome also persist (73). The complexity of the gut system and the variability in the environmental conditions pose many barriers in determining a causal relationship between habitat and the gut microbiome. However, future studies can subset by phylogenetic identity, life stage and feeding behavior and collect samples only from a certain time of year, as aquatic microbial communities are highly affected by seasonality, to further develop theories about the effects of habitat on fish gut microbiota (74).

Limitations This study contains several limitations, the most notable of which is the quality of Minich et al.'s dataset. The majority of fish species were only sampled once or twice per body site, allowing for poor to no resolution of the microbial dynamics of individual species. The lack of detailed water column samples to serve as a negative control renders us unable to discern whether we detected microbes because they are relevant to the microbiota of the fish or if it was because they are prevalent and abundant in the environment.

This is also compounded by the many confounding variables absent from the metadata collected by Minich et al. As previously discussed, abiotic factors known to impact the microbiome such as salinity, temperature, nutrient concentration, and luminance (75) were notably absent. Furthermore, characterization of basic biotic factors such as sex, a well-documented variable in causing differences in microbial compositions (76, 77), was also missing. These limitations make it difficult to determine if the root cause for our observations is in the dataset as various confounding variables may not have even been measured.

Choosing substrata as a point of focus in itself also proves to be a severe limitation. Minich et al. identified ten unique substrata within their metadata (5). However, these substrata were not sampled evenly, with certain substratum such as rocky shelf containing only a single sample. As such, only four substrata were represented after rarefaction, with only three being usable for downstream analysis of the midgut. Our results for substrata mirror their abundance. We resolved the most results for the sandy bottom substratum, but it was also the most abundant substratum for midgut and the whole dataset. There exists the possibility that this skew of data representation might have affected our ability to draw conclusions for other substrata. Coupled with the lack of a robust negative control, these limitations severely compromise the findings made in this paper.

Conclusions In this study, we explored the differences in ray-finned fish midgut microbiota from different substrata. We did not find that the midgut microbiota from samples originating from any substratum to be significantly different in abundance compared to any other substratum. From our core microbiome analysis, the core midgut communities from the three substrata are vastly different in terms of microbial composition, each having distinct core species, while sharing one common species, *Pseudoalteromonas sp.* Across the three substrata, we identified Proteobacteria, followed by Bacteroidetes and Firmicutes, to be the most dominant phyla in midgut core microbiomes. Midgut microbiomes from the sandy bottom substratum are characterized by indicator species *Synechococcus sp.* CC9902 and *Psychromonas sp.,* though the roles of the two species within the gut microbiota is unclear.

Overall, this study demonstrated that fish gut microbiota is mildly associated with substrata and provides the foundational knowledge in understanding how gut microbes regulate fish digestive and immune health, upon which the technology to develop fish probiotics and monitor population expansion in fish management (41) will be built from.

Future Directions It is unclear whether the dominant phyla are present in high abundance in the environment or if they have been selected by the host itself (69, 70). Future studies could subset by phylogenetic identity, life stage, sex, and feeding behavior and compare if the shared midgut species *Pseudoalteromonas sp.* and distinct phyla from different substrata still persist. The collection of water column samples as a negative control will also allow us to resolve whether the microbes we observed are truly part of the gut microbiome or are merely present in the environment. In addition, the relative abundance of Proteobacteria, Bacteroidetes, and Firmicutes are highly affected by seasonality. Future studies could control the season in which the samples are collected to ensure all samples have a consistent environmental baseline (74).

As the validity of this study has been compromised by a large number of confounding variables, we advise future studies to take on either of these two approaches. 1) A niche study aimed to eliminate confounding variables. Instead of comparing broadly across substrata, one could focus on the effects of different seasons on the fish gut microbiome in a single species of fish to standardize diet and phylogenetics, and taken from a consistent ocean depth to ensure the differences are seasonal instead of environmental (78). The results from a single well-controlled study could be broadly applicable to the fish gut microbiome overall. 2) A cross-sectional study with saturated sample size to determine overarching trends. One can compare how the fish gut microbiota changes with salt dependency, farmed fish, and infection (79, 80, 81, 82). Instead of controlling host-specific variables, the large sample size would account for the individual differences and shed light on the overarching trend of a dynamic fish gut microbiome.

With this knowledge, we can aim to manipulate conditions in aquacultures to promote fish growth, digestion and immune health (41). As the fish gut flora and fecal materials discharged into the water may reflect their diet preferences, physiological behaviors, and presence, gut microbiome research provides valuable information for monitoring fish invasion and population expansion that is important in fish conservation and management (41).

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