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# Comparison of the Gill Microbiome of Retail Oysters from Two Geographical Locations Exhibited Distinct Microbial Signatures: A Pilot Study for Potential Future Applications for Monitoring Authenticity of Their Origins

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**Abstract:** The oyster industry is a significant component of United States aquaculture and is vulnerable to various food frauds. In addition to species substitution, mislabeling of oyster geographical origin is performed for economic gains. The geographical origin misrepresentations are performed to claim a famed region of origin known for its unique flavor profile. DNA barcoding is the gold standard method for identifying seafood species but has limited resolution to the species level. This pilot study was conducted to characterize and compare the oyster gill microbiome as an alternative approach for tracking oysters' origin. Commercially available raw east coast oysters (*Crassostrea virginica*) from two distinct geographical locations were purchased. Genomic DNA isolated from the gills was processed for microbiome analysis. The data revealed distinct microbiome signatures among the two sample sets. Oysters from Louisiana showed the presence of eighteen unique bacterial genera, whereas Maryland oysters showed a higher abundance of twelve genera. Findings from this study demonstrate the applicability of microbiome analysis as an emerging alternative approach for identifying geographical origin misrepresentations.

**Keywords:** geographical origin; microbial ecology; microbiota; microflora; oyster; seafood



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## 1. Introduction

According to the National Oceanic and Atmospheric Administration (NOAA) 2018 report, the United States oyster landings were 30.3 million pounds, valued at USD 258.7 million [1]. In the United States, oyster production is led by the Gulf region, accounting for around 50% of the national total. The east coast oyster (*Crassostrea virginica*) has a vast geographical range that stretches along eastern North America from the Gulf of St. Lawrence to the Gulf of Mexico. They are the leading oyster species cultivated in this region. Based on salinity, temperature, and minerals associated with each geographical region, oysters have a distinct flavor. Thus, oysters belonging to the same species but from a different region of origin can have varying commercial value, when the cost is considered. In general, the more southerly the oysters are harvested, the lesser the value, which is due to the warmer Gulf water facilitating a continuous oyster growing season. The northeast oysters have a shorter growing season as cold water can lead to oyster dormancy. According to NOAA's 2021 data, Maryland oyster landing was 631 metric tons with a cost of USD 11.1/pound, whereas oyster landing from Louisiana was 2996 metric tons with a price of USD 7.8/pound [2]. Therefore, the northeast oysters from the famed cold-water areas (e.g., Bluepoint oysters, Island Creek) are sold at a premium due to low production, higher demand, and consumer taste preference.

Food misrepresentation was initially identified in the 1980s and still remains a major problem [3]. A meta-analysis estimated an average 8% mislabeling rate for seafood [4].

Oysters are a major seafood commodity; species and geographical origin misrepresentation have been reported for commercially available oysters [5], negatively affecting producers, restaurants, and stores selling authentic seafood products. Federal regulation entitles consumers to know the specific identity, production method, and geographical origin of the seafood [6]. The standard method of oyster species validation recommended by the U.S. Food and Drug Administration (U.S. FDA) includes the barcoding of the specific nucleotide sequence of the cytochrome oxidase I (COI) gene [7]. The barcoding method can only provide genus or species information. The method is limited in its ability to distinguish between samples of the same species harvested from two distinct geographical locations due to the lack of sequence variation in the COI genes of the samples from two locations [8]. In addition to seafood species misrepresentation, the fraudulent labeling of seafood's geographical origin is another critical challenge for the seafood industry. Seafood geographical origin misrepresentation is performed for economic benefit, where a product belonging to the same genus and species is deceptively claimed from a famed harvesting area with a higher price. In this case, traceability becomes challenging if the same seafood species are cultivated at multiple geographical locations, i.e., from the Gulf of St. Lawrence to Mexico. Past research in the area of inferring the geographical origin of seafood has focused on the identification of stable C and N isotopes and elemental analysis [9], and isotope ratio mass spectrometry analysis, which measures the ratios of 18O/16O to identify the geographical origin [10]. Additionally, microsatellites and single nucleotide polymorphisms (SNP) markers have been used to identify *Crassostrea virginica* from different regions [11,12].

Alternative methods for the seafood region of origin identification are needed as the consumer highly desires oysters, and oysters from famed areas considered a delicacy can easily be swapped with lower-quality or less expensive oysters from other regions [13].

Oysters interact closely with their surroundings and have site-specific microbial signatures [14,15]. Therefore, we hypothesized that oysters from a specific region exposed to a unique combination of geographical and climatic conditions and feed would possess a unique microbiome signature. As oyster gills are in constant contact with the surrounding water, this study aimed to characterize and compare the gill microbiome profiles of east coast oysters from two distinct geographical regions.

## 2. Materials and Methods

### *2.1. Sample Collection*

Oyster samples were collected from two distinct geographical locations in the United States (Figure 1). The northernmost oyster was farmed using a wire containment sack and was harvested on 25 January 2021 from St. Jerome Creek, MD, USA. These samples from Maryland were shipped with shells. Louisiana oysters were wild catch reef oysters, harvested on 27 January 2021 from GPS coordinates (29.997100, −89.301400) and grown at three feet ocean depth. This sample set was shucked before shipment. Each sample set comprised 36 oysters. Samples were shipped to the FSU food microbiology laboratory on ice. Both oyster samples were provided by J.J. McDonnell & Co. (Elkridge, MD, USA), a seafood wholesaler.

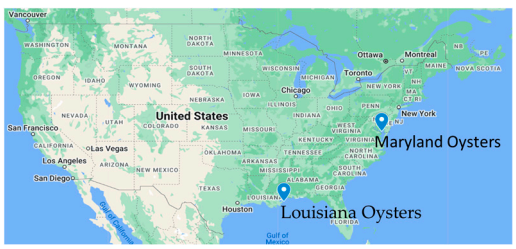


**Figure 1.** Two distinct sample collection locations are shown on the United States map.

### *2.2. Sample Processing and DNA Extraction*

Gills from the oyster samples were removed aseptically in a biosafety cabinet using sterile tools and plasticware. All the gill tissue from three oysters was pooled in one stomacher bag and diluted with 20 mL of sterile phosphate buffer saline (PBS) at pH 7.4 [16]. Thus, gills from 36 samples from each sample set resulted in 12 pooled samples. Samples were stomached at 230 rpm for 2 min to disrupt the oyster tissue and liberate bacterial cells. Two milliliters of homogenate from each stomacher bag were transferred to a sterile Petri dish. As collected homogenates had a high tissue content, homogenates were gently decanted to separate oyster tissue from PBS-containing bacterial cells. The PBS with bacterial cells was transferred to a 1.5 mL centrifuge tube, centrifuged at 15,000$\times$ *g* for two minutes to obtain a pellet, and was used for DNA isolation. DNA was isolated using the PowerFecal Pro DNA kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions.

### *2.3. Microbiome Analysis*

DNA samples were processed for microbiome analysis, as described in our previous studies [8,17,18]. To avoid any sample processing bias, all samples were simultaneously batch processed. Briefly, the Earth Microbiome Project (EMP) benchmarked protocol [19] was used to sequence and characterize the oyster microbiome. The microbial community analysis protocols were executed as previously described by Caporaso et al. [20]. Thus, the resulting 24 barcoded amplicons (i.e., 12 LA and 12 MD) were purified with Agencourt® AMPure® XP magnetic purification beads (Beckman Coulter, Brea, CA, USA). Purified samples were quantified using a Qubit fluorimeter (Qubit3; Invitrogen, Waltham, CA, USA). The purified and quantified PCR amplicons were pooled in equal molar concentrations and sequenced on one 2 $\times$ 300 bp Illumina MiSeq flow cell (Miseq reagent kit v3; Illumina Inc., San Diego, CA, USA) at FSU's molecular cloning facility. The sequencing quality control was executed through onboard Miseq Control Software and Miseq Reporter (Illumina Inc., San Diego, CA, USA). Sequencing reads (.fastq files) were de-multiplexed and quality trimmed and denoised with the high-resolution DADA2 inference workflow [21], using default parameters described in our previous studies [22]. Taxonomy classification was assigned to produced ASVs with Ribosomal Database Project (RDP)-classifier workflow as described by Wang et al. [23] using the QIIME2 software suite (version 2021.2) (Flagstaff, AZ, USA) [24] with the Greengenes-trained Naive Bayes classifier provided by QIIME2 (gg-13–8-99-nb-classifier.qza). Of a total of 3,397,737 reads originally obtained, 1,512,014 (mean 63,000 $\pm$ 5250) reads were obtained after quality filtering, adapter trimming, denoising, and removal of non-chimeric amplicons using DADA2's default parameters. Alpha-rarefaction was performed at the lowest sequencing depth, that is, 25,000 bp, to avoid the bias of sequencing depth. The dataset was filtered to exclude features annotated as 'mitochondria' and 'chloroplast'. To avoid the bias of sequencing errors or low-level contaminations, the ASVs (amplicon sequence variants) with a very small read count (less than four) in very few samples (less than 10% prevalence) were filtered out from the subsequent analyses. The data of the taxon relative abundance were further subjected to the total sum scaling, and the taxa with less than 1% mean relative abundance were excluded from the subsequent downstream analyses. All the samples were batch-processed identically and simultaneously to avoid the bias of nucleic acid extraction, PCR reaction, and primers on community composition obtained by amplicon sequencing. Alpha-diversity measures included ASVs, Chao1 (species richness), and Shannon (species evenness) index. Beta-diversity among two sample sets was computed using the principal coordinate analysis (PCoA) of the Bray–Curtis dissimilarity index as described previously [25]. Bacterial community composition was measured at the phylum and genus levels.

### *2.4. Data Analysis*

All data analysis was carried out using GraphPad (Prism version 9) and the 'R' statistical software package (version 4.0.3; https://www.r-project.org/ (accessed on 12 July 2021)). The statistical analysis for the differential clustering of samples on the PCoA plot was com-

puted by the PERMANOVA (permutational analysis of variance) test, a permutation-based multivariate analysis of variance to a matrix of pairwise distance to partition the inter-group and intra-group distance, using 9999 permutations. Intra-group variability in beta-diversity was estimated by applying a non-parametric analysis of similarities (ANOSIM) test of the Bray–Curtis ranked distance. The alpha-diversity indices and the proportions of specific bacterial taxa between different groups were compared using the two-tailed unpaired non-parametric Mann–Whitney test (95% confidence level). Volcano plots depicting the patterns of differential relative abundance of bacterial taxa were constructed within the 'R' package. LEfSE (linear discriminatory analysis [LDA] effect size) was used to identify bacterial taxa that drive differences between different oyster groups, with parameters set at an LDA score of more than 3.0 and an alpha-value of less than 0.01 [26]. The normalization method of taxon relative abundance data consisted of data transformation and scaling. Unless otherwise stated, all the values presented herein are means ± standard deviation. Statistical significance was set at $p < 0.05$ unless otherwise specified.

## 3. Results

In this study, using a high-throughput amplicon-sequencing approach, we characterized the gill microbiome diversity and composition of oysters collected from two distinct geographical regions, nearly the same date, and only one sampling time point.

The Figure 2 illustrates the beta-diversity (inter-group similarity) arrays of the gill microbiome in oysters from the Louisiana (LA) versus Maryland (MD) group, computed using the principal coordinate analysis (PCoA) ordination of the Bray–Curtis similarity index and compared using the PERMANOVA (permutational analysis of variance) and ANOSIM (non-parametric analysis of similarities) tests. As evident in Figure 2, the analyses clearly revealed distinct microbiome clusters in LA versus MD oysters, indicating that the two groups harbored significantly different gill microbiome community configurations (Figure 2a). In addition, further analysis of the intra-group Bray–Curtis distance demonstrated that the oysters in the MD group had relatively lower inter-individual and intra-group variability in the microbiome as compared with LA oysters (Figure 2b). Together, these data indicated a distinct gill microbiome configuration between these two groups of oysters.

To estimate the gill bacterial diversity in terms of richness of the microbiome community, we measured the alpha-diversity indices, viz. the observed number of ASVs, the Chao1 index (species richness), and the Shannon index (a measure of species evenness) in these two groups of oysters (Figure 2c–e). The analysis of these indices did not show any noticeable difference in the observed ASVs and Chao1 (richness) index. However, we noticed a higher Shannon index in oysters from MD versus the LA group, indicating that the microbiome community in the MD oysters was represented more evenly and/or by a greater number of taxa as compared with oysters from the LA group.

Subsequent analyses of the overall microbiome composition (Table S1) also demonstrated a distinct microbiome community composition between the two groups of oysters (Figure 3). In terms of the microbiome composition at the level of major bacteria phyla, we observed that the oysters in the MD group harbored a relatively higher relative abundance of the bacterial phyla *Tenericutes*, *Firmicutes*, and *Cyanobacteria* and a lower proportion of the phyla *Bacteroidetes*, *Spirochaetes*, and *Acidobacteria* as compared with the LA oysters (Figure 3a). Further deeper analysis at the level of major bacterial genera revealed a higher relative abundance of bacterial genera *Pseudoalteromonas* and *Vibrio* and a lower relative abundance of *Shewanella*, *Campylobacter*, and *Leptonema* in the MD versus the LA oysters (Figure 3b). Contrary to our expectations, the presence of Vibrios sequence reads in the MD oysters could be associated with cold-water Vibrios (*Aliivibrio salmonicida*, earlier known as *Vibrio salmonicida*), which typically grow when the water temperature is 10–15 °C [27]. Further, we observed that the ratio of Gram-positive to Gram-negative bacteria was significantly higher in the MD versus the LA group (Figure 3c).

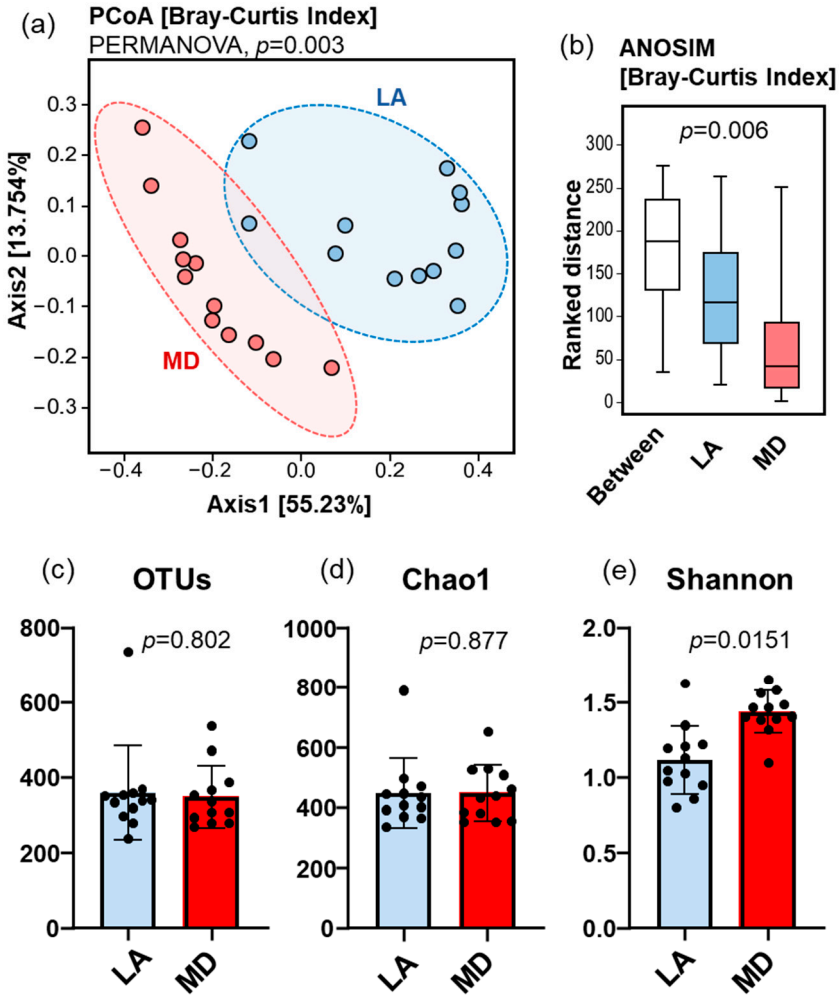


**Figure 2.** Distinct arrays of gill microbiome diversity in oysters from the two geographical locations. (**a**) Principal coordinate analysis (PCoA) showing clearly defined groups outlined by colored circles showing the 95% confidence intervals, (**b**) within-group variability of the Bray–Curtis distance, (**c**) the number of amplicon sequence variants (ASVs), (**d**) Chao1 index (species richness), and (**e**) Shannon index (species evenness) in oysters harvested from Louisiana (LA) versus Maryland (MD).

To further decipher which bacterial taxa were significantly different between the two groups of oysters, we conducted unbiased and discriminatory statistical analyses on the microbiome taxa detected. As seen in Figure 4a, the volcano plot analysis of the logarithmic difference in the relative abundance of bacterial genera revealed that the oysters in the LA group had a greater number of bacterial genera with a significantly higher proportion as compared with the oysters in the MD group. Subsequently, we executed the biomarker discovery algorithm, i.e., the linear discriminatory analysis (LDA) effect size (LEfSe) analysis, to identify unique bacterial taxa in each group. As indicated by the previous analyses, the LEfSe cladogram clearly demonstrated a distinct pattern of microbiome community between the two groups of oysters (Figure S1). Subsequent simplified analysis of bacterial genera based on the LDA score demonstrated that the oysters in the LA group had a significantly higher proportion of a total of eighteen taxa, including *Shewanella*, *Arcobacter*, *Campylobacter*, *Leptonema*, *Psychromonas*, *Flabovacterium*, *Desulfotomaculum*, *Pseudomonas*, *Mycobacterium*, *Desulfobacetrium*, *Aeromonas*, and *Sulfurospirillum*, whereas the MD group had a significantly higher relative abundance of twelve genera including *Pseudoalteromonas*, *Vibrio*, *Pilibacter*, *Photobacterium*, *Enterococcus*, *Spiroplasma*, *Enterovibrio*, and *Oceanispira* (Figure 4b).

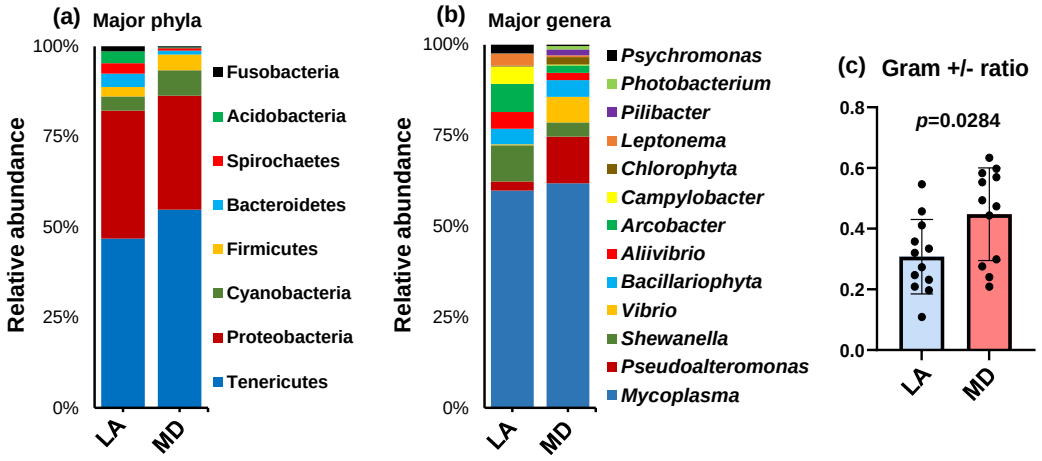


**Figure 3.** Gill microbiome composition differs between oysters from the two geographical locations. The microbiome composition at the level of (**a**) major phyla and (**b**) major genera and (**c**) ratio of Gram-positive to Gram-negative bacterial taxa between oysters harvested from Louisiana (LA) versus Maryland (MD).

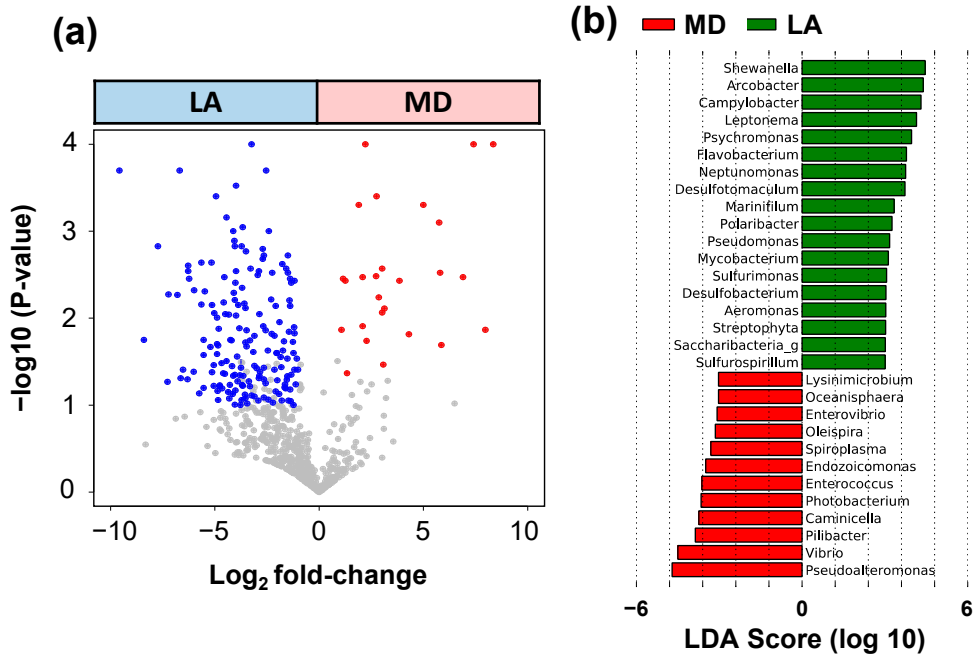


**Figure 4.** Distinct gill microbiome signatures in oysters from the two geographical locations. (**a**) Volcano plot illustrating the pattern of microbiome differences in terms of logarithmic difference in the relative proportion of bacterial taxa in oysters harvested from Louisiana (LA) versus Maryland (MD). Colored dots represent taxa that have relative abundance with Log2 fold difference >1.0 and $p$-value < 0.05 in comparison with that in the other group. (**b**) Linear discriminatory analysis (LDA) effect size (LEfSe)-based LDA score bar plot demonstrating unique microbial signatures in oysters harvested from Louisiana (LA) versus Maryland (MD).

## 4. Discussion

Fish and various fish products are some of the most widely traded food commodities worldwide. This rapid expansion in the fish trade has increased the world's fish consumption and associated cases of seafood fraud. Routing seafood products through a complex supply chain reduces traceability and creates an opportunity for seafood fraud [28,29]. Among different types of seafood frauds, the identification of geographical origin misrepre-

sentations is one of the most difficult to detect and thus is likely to go unnoticed, eventually hurting the profitability and credibility of seafood producers associated with a famed place.

Host-specific microbiome signatures can provide important ecological, evolutionary, and physiological clues as specific microbial communities can only inhabit specific ecological niches, e.g., soil, water, and animal tissues. Additionally, the microbiome of animal tissues is tightly regulated by the host's genetics, health, environment, diet, medication, and other extrinsic factors [30–32]. Hence, the characterization of microbial communities can reflect various host-specific features such as host environment and health pertaining to biotic and abiotic factors [33], which makes the microbiome a valuable tool for understanding host–microbe and host–environment interactions and geographical region of origin identification.

In a seminal work, King et al. [34] reported stomach and gut microbiota diversity for the eastern oysters collected from Louisiana (USA). The stomach microbiome of the oyster samples from Louisiana comprised *Spartobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Chloroflexi*, *Proteobacteria*, *Mollicutes*, *Firmicutes*, and *Mollicutes*. In comparison, the oyster gut microbiota was dominated by *Firmicutes*, *Mollicutes*, *Proteobacteria*, *Chloroflexi*, and *Verrucomicrobia* [34]. Interestingly, the oyster samples from Lake Caillou (Terrebonne Parish, LA, USA) showed overwhelming dominance of *Mollicutes* in the stomach samples, which was very similar to a high relative abundance of *Mycoplasma* in oyster samples collected in our study. Other studies reported a similar dominance of *Mycoplasma* in the Pacific oysters (*Crassostrea gigas*) [31,32,35,36]. Other studies on eastern oysters reported a predominance of *Cyanobacteria* (50–75%) [15,37]. Data from the abovementioned studies reflect that *Mycoplasma* and *Cyanobacteria* are dominant microbial communities of oysters and other bivalves [32,38].

The data generated in our study using one sample set collected from one site clearly demonstrate distinct clustering of samples collected from two regions (Figure 1a). Such geographical region-associated distinct microbiome signatures have been reported previously by us and others for oysters [34,37], shrimp [8], clams [29], Manila clams [39], sea bass [40], and salmon [41]. These unique signatures associated with seafood from a distinct geographical origin could be attributed to multiple interacting factors, which include diet [42], the host's digestive physiology [43], the coevolution of the host with the symbionts, and the distinct ecological condition associated with a geographical location [44].

Some of the limitations of the microbiome-based method for the identification of the geographical origin of oyster samples are the application of the depuration step by the industry to enhance the microbial safety of raw oysters. The depuration step facilitates the expulsion of the intestinal contents, facilitating the reduction of bacterial levels in the bivalve (i.e., clams, mussels, oysters, and cockles) [45]. Thus, the depuration step can result in a reduction of the microbiota. However, multiple samples can be pooled to increase the sensitivity of the method. To further standardize and validate the microbiome-based geographical origin method, a standard protocol for sample preparation, a data analysis pipeline, characterization of the oyster microbiome from various commercially important regions, and the creation of a repository of microbiome sequence data are needed.

This pilot study was part of our seafood microbiome initiative at Florida State University, where the microbiome composition of all samples (i.e., oyster and shrimp) was analyzed using the same method and database. Consistent with our previous finding showing distinct region-specific microbiome signatures among shrimp [8], this study demonstrates distinct gill microbiome signatures in oysters collected from two geographically distinct locations. Signature microbial species associated with samples from each geographical region can be used as markers for developing rapid PCR-based assays. However, these specific marker species associated with oysters from each location need to be extensively validated with multiple time point sampling from each location. This study has some limitations, including the small sample size, only two selected locations, one sampling point, and the use of shucked oysters. The shucking process may be responsible for a few microbiome profile differences. Additionally, as the study was performed using com-

mercially available oysters, we did not have access to water, sediments, and rock samples for analysis and comparison. Future studies should include microbiome characterization of the environmental samples, water temperature, and salinity, which will facilitate our understanding of the relationship between the oyster gill microbiome and its surroundings. Nevertheless, despite these limitations, this pilot study helps advance an emerging concept of using microbiota signatures as a potential tool to identify the region of origin, which is also applicable to other seafood species.

## 5. Conclusions

This pilot study demonstrates distinct microbiome signatures among samples collected from two different locations. The approach could be an alternative tool for identifying seafood's geographical region of origin. The study provides important information for seafood microbiologists, marine microbiologists, microbial ecologists, oceanologists, and food fraud experts. It builds a case for future studies with higher sample sizes and more diverse geographical locations for other major seafood specimens.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol3010001/s1, Figure S1: Linear discriminatory analysis (LDA) Effect Size (LEfSe) cladogram demonstrating unique microbial signatures in oysters harvested from Louisiana (LA) versus Maryland (MD).; Table S1: Raw data of relative abundance levels of microbial taxa detected in oysters harvested from Louisiana (LA) and Maryland (MD).

**Author Contributions:** Conceptualization, P.S.; methodology, P.S. and R.N.; software, R.N.; investigation, F.J.V.; resources, P.S. and D.W.; data curation, P.S.; writing—original draft preparation, P.S.; writing—review and editing, R.N. and D.W; supervision, P.S.; project administration, P.S.; funding acquisition, P.S. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The list of bacterial taxa detected has been provided as Supplementary Table S1. All the raw sequencing datasets have been submitted to the NCBI Sequence Read Archive (SRA) public repository database under SRA BioProject number PRJNA839104 (ncbi.nlm.nih.gov/sra/?term=PRJNA839104; ncbi.nlm.nih.gov/bioproject/PRJNA839104/).

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**Conflicts of Interest:** The authors declare no conflict of interest.

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