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Bacterial communities of wild-captured loggerhead sea turtles *Caretta caretta* in the Northwest Atlantic

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ABSTRACT: Investigating the gut microbiome of loggerhead sea turtles *Caretta caretta* provides insight into their health and foraging behavior. We analyzed the cloacal lavage microbiomes of 49 loggerhead turtles sampled from the Middle Atlantic Bight (MAB) and off North Carolina (NC) between 2021 and 2023. High-throughput sequencing of the 16S rRNA gene revealed that *Firmicutes*, *Fusobacteria*, *Proteobacteria*, and *Epsilonbacteraeota* were the dominant bacterial phyla. Microbial communities varied significantly based on sampling location and year. NC turtles sampled in March exhibited higher relative abundances of *Proteobacteria*, potentially linked to reduced foraging activity during overwintering. In contrast, MAB turtles sampled later in the season had higher microbiome diversity and shifts in dominant taxa, likely reflecting increased foraging. Seawater samples exhibited significantly different microbial communities from turtles, indicating limited immediate direct environmental influence on gut microbiome composition. These findings highlight the utility of cloacal lavages for microbiome studies and provide a baseline for future assessments of loggerhead turtle gut microbiota. As offshore wind developments and other anthropogenic pressures increase in the Northwest Atlantic region, continued microbiome monitoring will be a useful tool for evaluating potential impacts on sea turtle health and conservation.

KEY WORDS: Loggerhead · Sea turtles · Gut microbiome · *Caretta caretta* · Northwest Atlantic

1. INTRODUCTION

The gut microbiome plays a fundamental role in maintaining host health, contributing to development, digestion, metabolism, immune system regulation, and overall homeostasis (Sommer & Bäckhed 2013, Ogunrinola et al. 2020, Bidell et al. 2022). The composition of the microbiota is shaped by various factors, including diet, genetics, habitat, lifestyle, and disease (Sullam et al. 2012, Manor et al. 2020, Beam et al. 2021). Dysbiosis, or microbial imbalance, has been linked to a range of diseases including inflammatory conditions and metabolic disorders, underscoring the importance of maintaining a healthy microbiome (Gomaa 2020, de Vos et al. 2022). Monitoring microbiome composition has emerged as a tool for assessing not only individual health, but also ecosystem disturbances and population-level changes (Rocca et al. 2019, Ribas et al. 2023).

The sensitivity of the microbiome to environmental changes makes it a valuable indicator of ecological health of sea turtles, complementing traditional health monitoring methods such as blood biochemistry, pathogen surveillance, and physical assessments (Yang et al. 2019, Page-Karjian & Perrault 2021). Establishing a baseline for sea turtle gut microbiomes can help with large-scale population monitoring and conservation efforts (Egan & Gardiner 2016, Bloodgood et al. 2020). Determining their core microbiome allows for a better understanding of health and disease states (Ahasan et al. 2017, Apprill et al. 2017). This knowledge can also be applied to treatment and

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feeding protocols in rehabilitation facilities, where sea turtles are often cared for following stranding events (Innis et al. 2009, Bloodgood et al. 2020, McNally et al. 2021).

The loggerhead turtle *Caretta caretta* population in the Northwest Atlantic follows complex migratory patterns, with individuals traveling between overwintering areas in the southeastern USA and seasonal foraging grounds extending as far north as the Gulf of Maine (Winton et al. 2018). The Middle Atlantic Bight (MAB) is a crucial foraging ground that contributes to the stability of this population, especially for females that nest in North Carolina, South Carolina, and Georgia, USA (Pfaller et al. 2020). Seasonal migrations are influenced by oceanographic conditions and resource availability, with evidence suggesting that climate change will alter these movement patterns and increase interactions with northern fisheries (Patel et al. 2021). Loggerhead turtles in the Northwest Atlantic face numerous threats, including fisheries bycatch, coastal development, ecosystem alterations, disease, and climate change (Haas 2010, Bolten et al. 2011, Warden et al. 2015). Fisheries interactions have historically been among the greatest threats to loggerhead turtles, contributing to high mortality rates (Wallace et al. 2011). Habitat degradation from human activities, including those from marine operations and developments, further exacerbate conservation challenges (Fuentes et al. 2023). The International Union for Conservation of Nature (IUCN) lists the loggerhead turtle as Vulnerable, highlighting the importance of monitoring these populations (Casale & Tucker 2017).

Sea turtles are keystone species that provide numerous benefits to both marine and terrestrial ecosystems (Lovich et al. 2018). Despite their ecological significance, sea turtle microbiomes remain relatively understudied. Investigations into their gut microbiomes indicate that microbial communities are influenced by diet, geographic location, life stage, and health status (Ahasan et al. 2018, Campos et al. 2018, Scheelings et al. 2020b). Loggerhead turtle gut microbiomes commonly include the bacterial phyla *Firmicutes* (also known as *Bacillota*), *Bacteroidetes* (also known as *Bacteroidota*), *Proteobacteria* (also known as *Psuedomonadota*), and *Fusobacteria*, regardless of sampling methods, geographic location, and health status of turtles (Abdelrhman et al. 2016, Arizza et al. 2019, Biagi et al. 2019, Scheelings et al. 2020b, Forbes et al. 2023). Loggerhead turtle microbial communities appear distinct from those of the surrounding environment (e.g. tank water) (Biagi et al. 2019, Filek et al. 2021).

We aimed to characterize the gut microbiome of loggerhead turtles in the Northwest Atlantic region and explore how factors such as geographic location and temporal variation are related to microbial diversity. The bacterial communities of 49 loggerhead sea turtles from different locations and years in the Northwest Atlantic were examined via cloacal lavage samples. The objective was to establish baseline microbiome data for from healthy wild-caught loggerhead turtles (Yang et al. 2019) and seawater samples in the region. This research contributes to a growing body of work that could inform conservation and rehabilitation strategies, ultimately improving the management of this Vulnerable species.

2. MATERIALS AND METHODS

2.1. Sample collection

The study involved cloacal lavage samples from 49 wild-caught loggerhead sea turtles collected from late May 2021 to late June 2023. Turtles were captured in the MAB during May to June each year and off the coast of North Carolina (NC) in early March 2023 (Fig. 1). These turtles and samples were collected under US Endangered Species Act (ESA) permits #23639 and #22218.

Loggerhead turtles were captured using methods first described by Patel et al. (2018). Turtles were captured using a net, then transferred to a larger boat for processing. The minimum curved carapace length (CCL), measured from notch to notch, and body weight were recorded for each turtle (Table 1). Turtles were then placed on rubber tires to elevate them and facilitate access to the cloaca for sample collection. Turtles were restrained by hand and a sterile catheter tube (size 18Fr) was gently inserted into the cloaca and advanced to a depth sufficient to potentially reach the rectum. Sterile saline (0.9%) was flushed through the catheter into the cloacal cavity until fecal material was returned. Lavage fluid and any fecal material expelled were collected in a sterile bin beneath the turtle, then transferred to 50 ml tubes which were promptly stored on ice. The lavage collection bin was sterilized with a 10% bleach solution between each turtle. Total lavage volumes, including saline and fecal material, were recorded from 2022 onward; however, this measurement was not recorded for the 2021 sampling period. The average lavage volume for each turtle was 15.7 ml (0.23 ml per kg of body weight), with a minimum volume of 3.5 ml and a maximum volume of 51 ml (Table S1 in the Supple-

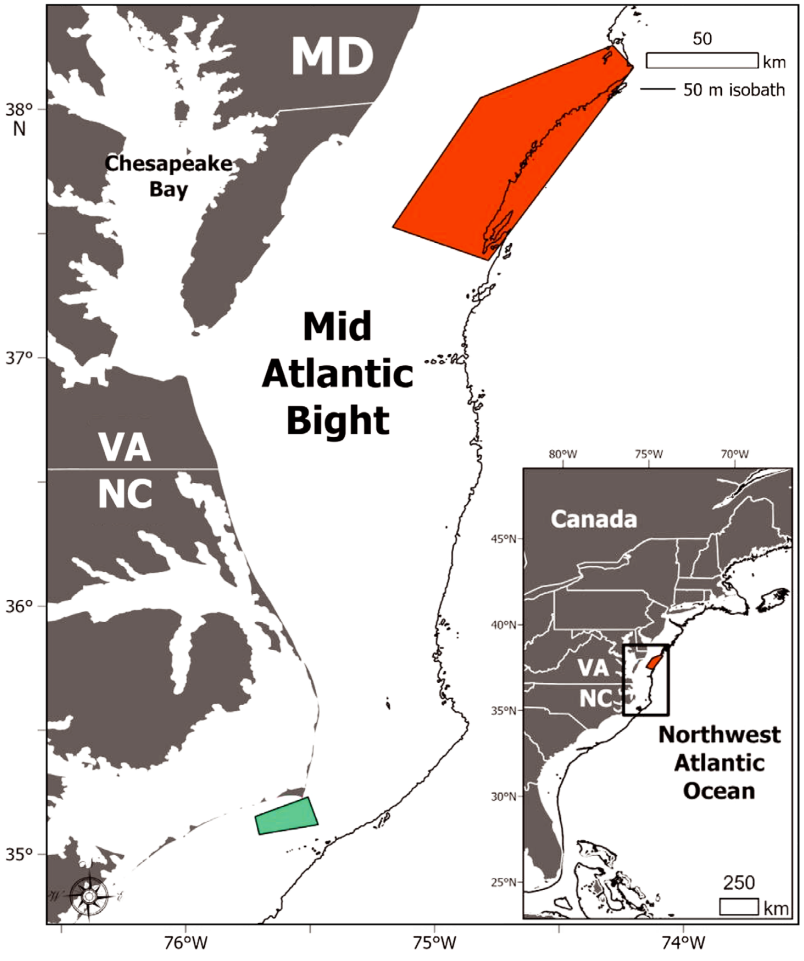


Fig. 1. Sampling locations for loggerhead turtles in the Northwest Atlantic Ocean. Turtles captured in the Middle Atlantic Bight are bounded by an orange polygon, while turtles captured off North Carolina are bounded by a green polygon. Inset map with a black box showing overall sampling area. MD: Maryland; VA: Virginia; NC: North Carolina

ment at www.int-res.com/articles/suppl/esr01547_supp.pdf).

Environmental water samples (1 l) were collected in 2023 from the MAB (n = 3) and NC (n = 2) at the same locations where turtles were captured, using sterile water bottles attached to a dip stick that were submerged into the ocean surface. Lavage and water samples from the MAB stayed on ice for up to 6 d at sea before being transferred to a 4°C refrigerator for storage until extraction. From NC, within 3–5 h of collection, lavage samples were refrigerated at 4°C and water samples were frozen at −20°C until extraction.

2.2. DNA extraction

DNA extraction was performed following the protocol described by Forbes et al. (2023) for fecal samples, with slight modifications. Briefly, lavage samples were homogenized, and 200 µl were collected from each sample for extraction. DNA extraction was performed using the GeneMATRIX™ Tissue DNA Purification Kit (EURx), following the manufacturer's protocol for liquid tissues, with alterations that included a 30 min incubation with the lysis buffer (Sol T) at 70°C, with samples vortexed at 10 min intervals, and a reduced final elution volume of 25 µl compared to the recommended range of 50–150 µl.

Water samples were filtered via vacuum filtration using a 0.22 µm membrane filter (Wilkem Scientific). Filters were folded and transferred with sterile forceps into PowerWater® Bead Pro Tubes for extraction. The DNeasy® PowerWater® kit (Qiagen) was used with slight modifications, including (1) a 10 min incubation with the lysing agent (Solution PW1) at 65°C, and (2) an elution volume of 25 µl instead of the recommended 100 µl.

Once extracted, DNA quality and quantity were assessed using a NanoDrop™ One^c spectrophotometer (Thermo Scientific). All extracted DNA was stored at −20°C prior to PCR amplification and then long-term at −80°C.

2.3. PCR amplification and sequencing

The hypervariable V1–V3 regions of the bacterial 16S rRNA gene were amplified via PCR following the protocol of Ahasan et al. (2017). Each reaction (50 µl total volume) contained 25 µl of AmpliTaq Gold 360 Master Mix (Life Technologies), 5 µl of template DNA, and 0.2 µM of each primer: 27F 5′-AGA GTT

Table 1. Demographic information for loggerhead turtles captured in the Middle Atlantic Bight (MAB) and North Carolina (NC); CCL: curved carapace length (notch to notch). Values are mean ± SD

Group	Sample size	CCL (cm)	Weight (kg)
MAB	40	90.1 ± 9.4	98.3 ± 30.2
NC	9	73.3 ± 9.1	54.8 ± 18.7

TGA TCM TGG CTC AG-3′ and 519R 5′-GWA TTA CCG CGG CKG CTG-3′. Primers included Illumina adapter overhang sequences (Klindworth et al. 2013). Amplification was performed on a T100™ Thermal Cycler (BioRad) with an initial denaturation at 95°C for 7 min; followed by 29 cycles at 94°C for 45 s, 50°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 7 min (Ahasan et al. 2017, Forbes et al. 2023). Resulting amplicons were submitted to the Rhode Island Genomics and Sequencing Center for library preparation and sequencing on an Illumina MiSeq™ using a 600 cycle Reagent Kit v3.

2.4. Data analysis

To ensure high-quality sequence data for downstream analysis, raw reads underwent several processing and filtering steps. De-multiplexed paired-end sequences were processed using QIIME2 (version 2023.2.0; Bolyen et al. 2019). The DADA2 pipeline (version 1.26) was implemented for primer removal, quality filtering, and merging of paired-end reads (Callahan et al. 2016). Primer sequences (20 bp forward, 18 bp reverse) were trimmed, and reads with a quality score of <20 were excluded. Additional filtering removed low-frequency features (≤400 reads) to reduce the influence of potential sequencing errors in downstream analysis. After initial processing with DADA2, sequences were denoised and resolved into amplicon sequence variants (ASVs). Representative sequences were then used for taxonomic assignment with a SILVA 132 classifier trained on the 16S rRNA V1–V3 region at 97% similarity (Quast et al. 2013). This threshold was chosen to approximate species-level classification while accounting for sequencing errors and biological variation of the 16S rRNA gene. Taxonomic names reflect the SILVA 132 database; more recent nomenclature updates were not retroactively applied to maintain consistency with classifier outputs. To standardize sequencing depth across samples and minimize bias, rarefaction analysis was conducted to determine an appropriate sequencing depth. Rarefaction analysis was performed by plotting the number of observed ASVs versus the total number of filtered reads for each sample. Based on the rarefaction curves, a rarefaction depth of 400 was selected to retain the maximum number of samples while ensuring adequate sequencing coverage. Following rarefaction, relative taxonomic abundances were visualized using taxa bar plots generated in QIIME2 from the taxonomically assigned feature table. A heatmap was generated after collapsing the feature table to the family level to visualize relative abundances of dominant bacterial families among sampling groups.

2.4.1. Alpha diversity

Data were imported into R version 4.3.2 (R Core Team 2023) using the 'readr' package (Wickham et al. 2024), then organized for analysis using the 'dplyr' package (Wickham et al. 2023a). Alpha diversity metrics were calculated to evaluate and compare microbial diversity among turtle and water sample groups. Alpha diversity was quantified using the Shannon-Weiner index and Faith's phylogenetic diversity. Because the data were not normally distributed, based on visual inspection of quantile-quantile plots and results from Shapiro-Wilk tests conducted in R, pairwise comparisons were performed using Wilcoxon rank-sum tests with Holm-adjusted p-values to account for multiple comparisons. Boxplots were created using the 'ggplot2' package in R (Wickham 2016) to show the distribution of diversity indices for each of the sampling groups. Linear mixed models (LMMs) were used to assess the relationship between alpha diversity and turtle size, with CCL as a fixed effect and turtle group (sampling location and year) as a random effect to control for variation between groups. The significance of CCL as a fixed effect was evaluated by comparing models to a corresponding null model using likelihood ratio tests. LMMs were implemented using the 'lme4' and 'MuMIn' packages in R (Bates et al. 2015, Bartoń 2024).

2.4.2. Microbiome composition

To evaluate differences in microbial community composition between sample groups, beta diversity analyses were conducted. ASV abundances were compared across sample types (water vs. turtle) and among turtle groups, which were categorized by year and sampling site. Specifically, turtle samples were grouped as 2021 MAB, 2022 MAB, 2023 MAB, and 2023 NC, while water samples were categorized as MAB water and NC water. Differences in microbial community composition among these groups were compared using a permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, implemented using the 'vegan' package in R (Oksanen et al. 2022). Beta diversity between sampling groups was visualized using a non-metric multidi-

mensional scaling (NMDS) plot of the Bray-Curtis dissimilarity metric, also using 'vegan'. Generated figures were exported as svg files using the 'svglite' package (Wickham et al. 2023b). A public repository containing all QIIME2 and R scripts used for statistical analyses and figure generation is available at https://github.com/Zachary-Forbes/Loggerhead-sea-turtle-gut-bacterial-communities.

3. RESULTS

3.1. Turtle information

Mean (±SD) CCL was 90.1 ± 9.4 cm for all turtles captured in the MAB, and 73.3 ± 9.1 cm for turtles captured in NC. Mean body weight was 98.3 ± 30.2 kg for MAB turtles and 54.8 ± 18.7 kg for NC turtles (Table 1).

3.2. Sequencing results and data analysis

Illumina MiSeq™ sequencing of the V1–V3 region of the 16S rRNA gene produced 13 775 627 raw reads from 49 turtle and 5 water samples. Sequences were submitted to the NCBI Sequence Read Archive (BioProject Accession #PRJNA1240848). Following filtering and chimera removal, 310 617 high-quality, merged reads remained. Samples contained an average of 5355 sequences, with read lengths ranging from 329 to 476 bp, and a mean length of 460 bp. These sequences were clustered into 67 distinct ASVs, which were classified to the species level and encompassed 12 phyla, 17 classes, and 44 families. ASVs that could not be taxonomically classified were designated as 'Unclassified'.

3.3. Alpha diversity

Alpha diversity, measured by Shannon's index and Faith's phylogenetic diversity, was mostly similar across turtle groups, though turtles captured in NC had the lowest mean diversity values by both metrics. Means ranged from 3.39 to 4.71 by Faith's phylogenetic diversity and 2.20 to 2.91 by Shannon's index. Pairwise Wilcoxon rank-sum tests with Holm-adjusted p-values indicated that loggerhead turtles captured in the MAB in 2023 exhibited significantly higher Faith's phylogenetic diversity compared to those captured in the MAB in 2022 ($p = 0.035$) and NC turtles captured in 2023 ($p = 0.031$). No significant differences in Shannon diversity were observed across any turtle groups ($p > 0.05$) (Fig. 2).

Sea water samples exhibited higher mean alpha diversity values for both Shannon and Faith's indices compared to the loggerhead groups. Water from the MAB had significantly higher Faith's phylogenetic diversity than MAB turtles from 2021 ($p = 0.038$). Additionally, MAB water samples had significantly higher Shannon diversity compared to MAB turtles from 2021 ($p = 0.044$) and 2023 ($p = 0.044$). Sea water samples from NC showed similar diversity values to MAB water by both metrics (Fig. 2).

CCL (cm) was positively associated with alpha diversity in linear mixed models. For Faith's phylogenetic diversity, CCL was a significant positive predictor ($\beta = 0.0253\ cm^{-1}$, SE = 0.0115). A likelihood ratio test indicated that including CCL significantly improved model fit when compared to the null model ($\chi^2 = 4.97$, df = 1, $p = 0.026$). The fixed effect explained 9.57% of the variance (marginal $R^2 = 0.0957$). Similarly, CCL was a significant positive predictor of Shannon diversity ($\beta = 0.0306\ cm^{-1}$, SE = 0.0095). CCL also significantly improved model fit ($\chi^2 = 9.78$, df = 1, $p = 0.0018$), explaining 17.9% of the variance (marginal $R^2 = 0.1786$) (Fig. 3).

3.4. Microbiome composition

Among the turtles captured in the MAB in 2021, the phylum *Fusobacteria* was the most abundant, with a mean relative abundance of 39.90% (Fig. 4). This was followed by *Proteobacteria* (30.53%), *Firmicutes* (11.73%), and *Epsilonbacteraeota* (also known as *Campylobacterota*) (10.60%). At the family level, *Fusobacteriaceae* (35.55%), *Rhodobacteraceae* (21.28%), and the *Clostridiales vadinBB60* group (11.56%) were the most prevalent taxa (Fig. 5).

In contrast, MAB 2022 turtles had microbiomes dominated by *Firmicutes* (60.82%), followed by *Fusobacteria* (15.23%) and *Lentisphaerae* (11.75%) (Fig. 4). The major families in this group were the *Clostridiales vadinBB60* group (59.98%) and *Fusobacteriaceae* (12.09%) (Fig. 5).

For turtles captured in the MAB in 2023, *Epsilonbacteraeota* was the most abundant phylum (51.73%), followed by *Fusobacteria* (14.67%) and *Proteobacteria* (11.93%) (Fig. 4). The dominant families were *Sulfurospirillaceae* (30.00%), *Arcobacteraceae* (16.95%), *Leptotrichiaceae* (12.96%), and *Rhodobacteraceae* (11.32%) (Fig. 5).

Turtles captured in NC in 2023 had microbial communities heavily dominated by *Proteobacteria*

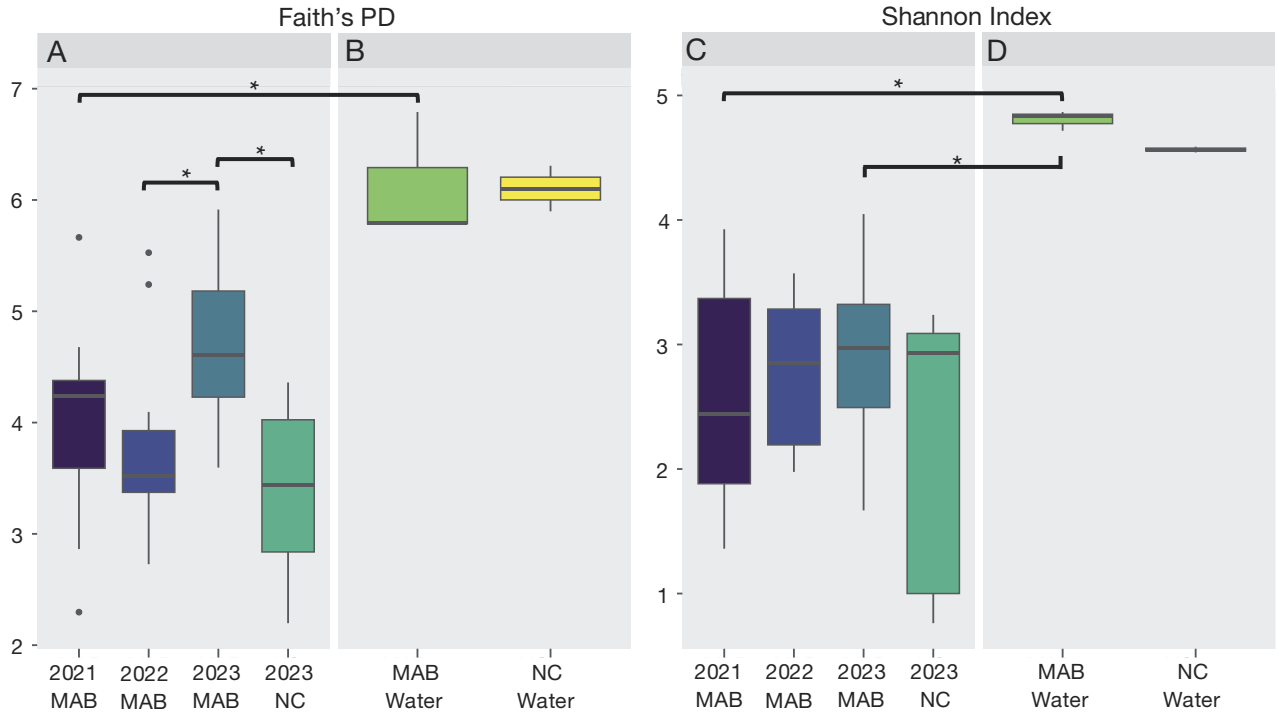


Fig. 2. Alpha diversity, measured by Faith's phylogenetic diversity (A, B) and Shannon index (C, D), comparing bacterial communities in loggerhead turtle and water samples. Turtles are grouped by sampling location and year, and water samples are grouped by location. Asterisks denote statistically significant differences identified by pairwise Wilcoxon rank-sum tests. Boxes indicate the first to the third quartile, with the median shown as a central line. Whiskers represent the full range of values, and points represent outliers. MAB: Middle Atlantic Bight; NC: North Carolina

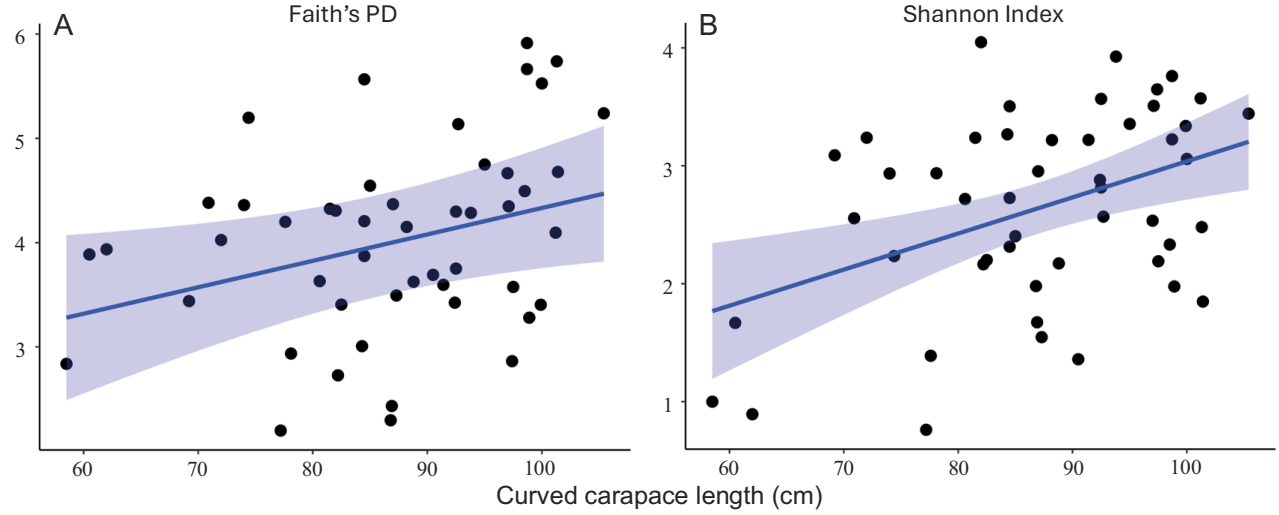


Fig. 3. Relationship between alpha diversity, measured by Faith's phylogenetic diversity (A) and Shannon index (B), and curved carapace length (CCL) in loggerhead sea turtles from all groups. Black points represent individual turtles, while the blue regression line illustrates the predicted relationship based on a linear mixed model (LMM), accounting for the random effects of sample group. Shaded blue areas indicate 95% confidence intervals. Both diversity metrics show a significant positive relationship with CCL (Faith's PD: r = 0.31, R^2 = 0.0957; Shannon: r = 0.42, R^2 = 0.1786)

(69.15%) and *Firmicutes* (22.25%), with lower abundances of *Epsilonbacteraeota* (2.66%) and *Spirochaetes* (2.17%) (Fig. 4). At the family level, the most prevalent taxa were *Rhodobacteraceae* (69.15%), the *Clostridiales vadinBB60* group (21.09%), and *Brachyspiraceae* (2.17%) (Fig. 5).

The microbial composition of MAB water samples was dominated by *Proteobacteria* (81.68%), followed

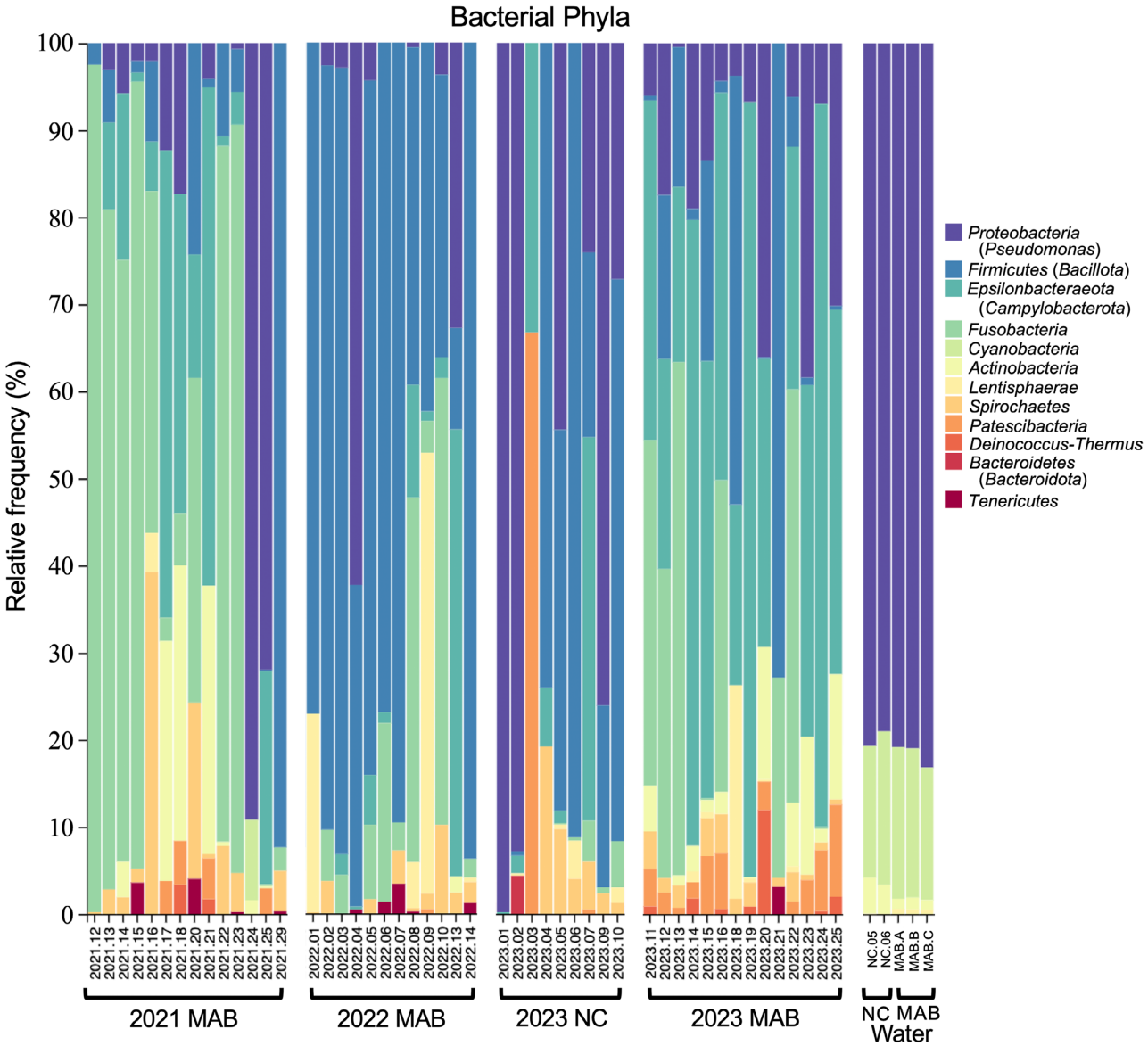


Fig. 4. Composition of loggerhead turtle cloacal lavage microbiomes, showing relative abundances of bacterial phyla across individual turtle samples, organized by sampling location and year. Each column on the chart represents an individual turtle. NC: North Carolina; MAB: Middle Atlantic Bight

by *Cyanobacteria* (16.55%), and *Actinobacteria* (1.22%). Water from NC showed a similar composition with only slight differences in relative abundance, being comprised of *Proteobacteria* (80.08%), *Cyanobacteria* (16.04%), and *Actinobacteria* (3.85%) (Fig. 4). At the family level, MAB water samples were dominated by Clade I within the SAR11 clade (*Alphaproteobacteria*; 39.95%), *Rhodobacteraceae* (15.04%), and *Cyanobiaceae* (11.48%). NC samples showed a similar dominance of Clade I SAR11 (49.49%), *Rhodobacteraceae* (9.87%), and *Cyanobiaceae* (9.84%) (Fig. 5).

When assessing the beta diversity of each sample group (categorized by collection year and location), ASV abundances were compared using Bray-Curtis dissimilarity to determine the degree of variation in microbial composition among groups at the phylum level (Fig. 6). PERMANOVA revealed a significant effect of sample group on microbial composition among loggerhead turtles ($F_{3,45} = 3.863$, $R^2 = 0.2048$, $p < 0.001$), indicating that composition differed among groups. Among MAB turtles, microbial composition differed significantly by year ($F_{2,37} = 4.697$, $R^2 = 0.2025$, $p < 0.001$). When all turtle groups were compared to the sea water groups, microbial

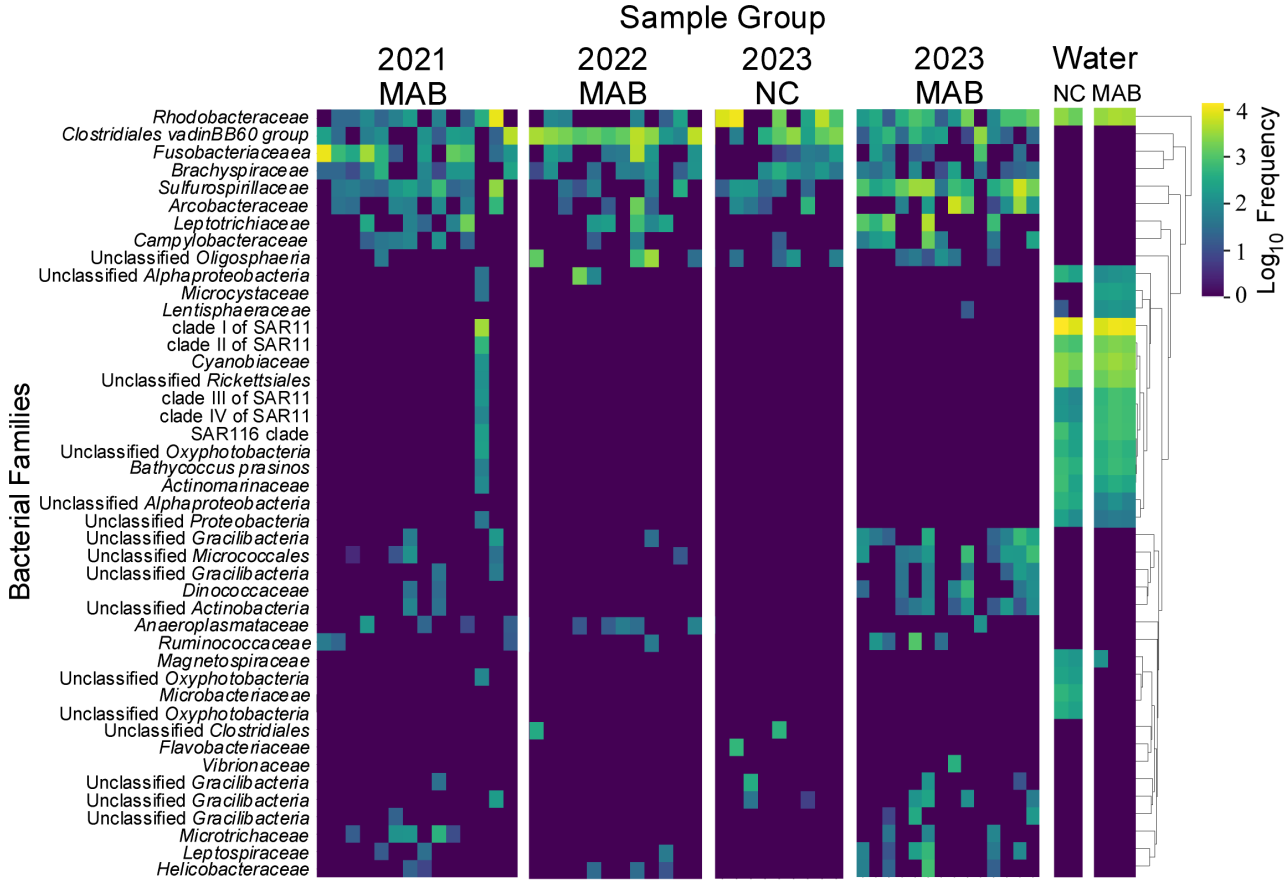


Fig. 5. Relative abundances of the 44 most prevalent bacterial families across turtle samples. Turtles are grouped by sampling location and year, and water samples are grouped by location. Individual turtles are labeled for this heatmap in Fig. S1 in the Supplement. NC: North Carolina, MAB: Middle Atlantic Bight

composition differed significantly between sample types ($F_{1,52} = 7.881$, $R^2 = 0.1316$, $p < 0.001$).

4. DISCUSSION

The choice of sampling method is a critical consideration in microbiome studies, particularly for sea turtles where access to *in situ* gastrointestinal (GI) tract samples is limited. Historically, most studies of live sea turtles have relied on fecal samples or cloacal swabs as proxies of the gut microbiome, each presenting distinct advantages and limitations (Kuschke 2022). Fecal samples may be more representative of the entire GI tract due to their inclusion of microbial communities from multiple gut regions (Ahasan et al. 2020, Bloodgood et al. 2020, Forbes et al. 2023). However, obtaining fecal matter from live, wild-caught turtles can be inconsistent and may require prolonged observation or handling (Price et al. 2017). In contrast, cloacal swabs are minimally invasive and obtained relatively quickly but primarily capture the microbiota of the cloacal mucosa, which may be influenced by environmental exposure and aerobic conditions (Price et al. 2017, Forbes et al. 2023). The cloacal microbiome is not necessarily representative of the proximal GI tract and may exclude the influence of parasites on local microbiota, which commonly reside in the stomach or upper intestine of loggerhead turtles (Pace et al. 2019). Cloacal lavage, as employed in this study, represents an intermediate approach by combining elements of both sampling methods. This technique returns a mixed sample that can contain both cloacal mucosa-associated microbes and fecal material from the distal intestine. However, the relative contribution of these components is variable and likely influences the resulting microbial community profile. Microbiome composition differs between fecal and cloacal communities within the same individual for loggerhead, green, and Kemp's ridley turtles (Price et al. 2017, Forbes et al. 2023). Additionally, microbial communities have been shown

to vary along different regions of the GI tract in green turtles (Ahasan et al. 2020, McDermid et al. 2020), further underscoring that sampling method can influence observed community structure.

In the present study, all cloacal lavage samples contained some degree of fecal material, although the quantity varied among samples. This variation likely contributes to heterogeneity in microbial composition, with samples containing greater fecal content potentially more closely reflecting the fecal or distal GI microbiome, while those with minimal fecal material may be more comparable to cloacal swabs. As such, differences in fecal content represent an important source of variability that should be considered when interpreting the data. Prior to DNA extraction, samples were homogenized to promote even distribution of microbial material; however, given the variable content of fecal material within lavage samples, some degree of within-sample heterogeneity is likely. Future studies including technical replicates of lavage samples would be valuable for assessing variability of lavage microbiome profiles. Using a standardized volume of sterile saline for each lavage may also facilitate sample comparison, along with recording descriptions of sample appearance and amount of fecal material present.

Potential environmental contamination is an important consideration when using cloacal lavage in field settings. The cloaca is a non-sterile site, and external sterilization was not performed in this study. While some protocols have incorporated pre-sampling cleansing with alcohol in an attempt to reduce external contamination (Price et al. 2017, Ahasan et al. 2018), such practices are not always applied and may not effectively eliminate microbial presence. The cloacal microbiome may be inherently influenced by environmental exposure, as turtles are in constant contact with surrounding seawater (Price et al. 2017, Forbes et al. 2023). Therefore, incidental inclusion of environmental or seawater-associated taxa cannot be fully excluded. In the present study, 1 turtle sample exhibited a microbial profile more similar to surrounding seawater, including the presence of *Cyanobacteria*, which were otherwise absent in turtle samples but consistently detected in water samples. However, the overall significant separation between turtle and water bacterial communities suggests that such contributions were limited, consistent with previous findings (Biagi et al. 2019, Filek et al. 2021). Future applications of cloacal lavages should consider incorporating environmental and negative controls to better assess potential sources and extent of contamination.

Despite these limitations, cloacal lavages remain a practical and minimally invasive sampling method that can potentially capture a broader representation of the gut microbiome than cloacal swabs, while being more feasible to obtain than fecal samples in wild populations. In addition to community-level analyses, cloacal lavage has demonstrated utility for the detection of specific bacterial taxa, including pathogens. For example, this method has been used to identify *Leptospira* spp. in freshwater turtles (Oliveira et al. 2016, Freitas et al. 2024). Several bacterial groups associated with the gastrointestinal tract of sea turtles, including members of the family *Enterobacteriaceae* (including *Salmonella* spp.) and the genera *Vibrio* and *Enterococcus*, include taxa of clinical relevance (Ebani 2023), and may be detectable using cloacal lavage-based sampling. In the present study, *Vibrio ordalii*, a known pathogen of salmonids (Echeverría-Bugueño et al. 2020), was detected in a single individual. Although the significance of this species in sea turtle health is undetermined, its detection highlights the potential for cloacal lavage to recover taxa of potential pathogenic relevance while simultaneously characterizing overall microbial community structure.

Collecting samples from loggerhead turtles across 3 yr and 2 distinct geographic locations allows for unique comparisons. Significant differences in relative phyla abundance were observed among groups, reflecting variation in community composition. Variation in sea turtle gut microbiomes have been associated with multiple ecological and host-related factors. Studies of wild populations have demonstrated that geographic location, life stage, and habitat changes influence microbiome composition (Price et al. 2017, Campos et al. 2018, Scheelings et al. 2020b). Rehabilitating turtles have shown shifts in microbial communities over time, suggesting that changes in diet and health status also play important roles (Ahasan et al. 2018, Bloodgood et al. 2020, Samuelson et al. 2020, McNally et al. 2021). Differences observed between NC and MAB turtles were therefore expected, as geographic location influences loggerhead microbiome composition, likely through differences in host genetics and foraging habitats (Scheelings et al. 2020b). Turtles sampled in this study may have originated from different nesting populations and exhibit fidelity to specific foraging grounds (Evans et al. 2019, Pfaller et al. 2020, Ceriani et al. 2023), which could further influence microbiome composition. It should also be noted that differences in sample handling occurred between locations, with NC samples refrigerated for several hours prior to freezing, where-

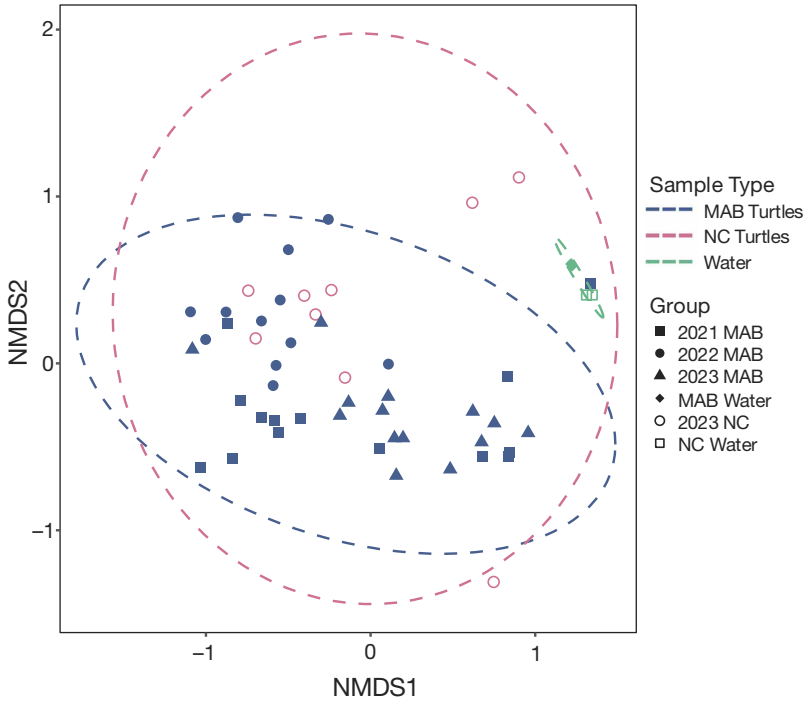


Fig. 6. Non-metric multidimensional scaling (NMDS) analysis illustrating differences in bacterial community composition between turtles and water samples. Dashed ellipses represent the 95% standard deviation ellipses of each sample type based on Bray-Curtis dissimilarities. Turtles are grouped by sampling location and year, and water samples are grouped by location. Each symbol represents an individual turtle, while color indicates sample type and shape represents sampling group

as MAB samples were placed on ice immediately, potentially allowing post-collection bacterial proliferation and contributing to the observed differences. Notably, even within the MAB, where turtles were sampled from the same general region, microbiome composition varied significantly between years, suggesting that interannual environmental variability likely contributed to these differences. Some of this variation may be driven by changes in prey availability, as sea turtle microbial communities can respond rapidly to shifts in diet in the wild (Price et al. 2017, Campos et al. 2018). Additionally, substantial variability was observed among individuals within groups. This may reflect preferential foraging behavior, as loggerhead turtles are generalist carnivores that consume a wide range of prey and can develop individual dietary preferences (Bjorndal 1997, Frick et al. 2009, Vander Zanden et al. 2010). The high variation between sampling groups indicates a need for increased sampling, with attempts to make correlations between changes in microbiome composition and specific environmental or anthropogenic factors.

Behavioral patterns may have contributed to the microbiome differences observed between sample groups. Turtles inhabiting NC nearshore waters in March typically exhibit brumation behavior of localized movement patterns and few extremely long dives per day, while in the MAB during May and June, turtles are actively migrating and taking many short dives as they transition from their wintering site to their summer foraging grounds (Hawkes et al. 2007, Winton et al. 2018, Braun McNeill et al. 2020, Hatch et al. 2022). Although turtles captured in NC were foraging, as indicated by the presence of fecal matter in the cloacal lavage samples, the frequency of their feeding and the potential prey items were likely different than that of the more active turtles captured in the MAB later in the season. These differences in foraging and movement patterns could result in distinct dietary inputs, which can directly affect microbial composition (Bloodgood et al. 2020). As a result, it is important to consider the prevailing behavior phase, physiological state, and geography when contextualizing these results (Scheelings et al. 2020b). Although alpha diversity remained relatively consistent across groups, turtles from NC had the lowest alpha diversity values by both metrics and were significantly lower than turtles captured in the same year in the MAB by Faith's phylogenetic diversity. The observed changes in alpha and beta diversity align with the understanding that factors such as geographic location and diet play distinct roles in altering gut microbial communities in sea turtles (Price et al. 2017, Campos et al. 2018, Scheelings et al. 2020b).

Relative abundance of *Proteobacteria* (also known as *Pseudomonadota*) in particular may highlight this relationship between foraging behavior and microbiome composition. *Proteobacteria* were found in higher relative abundance in NC turtles compared to MAB turtles across all years. Within MAB turtles, those sampled earlier in the foraging season (late May 2021) exhibited the highest levels of *Proteobacteria*, while those sampled latest in the foraging season (late June 2022) had the lowest levels. Previous research by Scheelings et al. (2020a) indicated that *Proteobacteria* dominate the distal colon of healthy adult loggerhead turtles undergoing periods of prolonged inappetence while nesting. In green turtles, *Proteobacteria* levels were higher in stranded turtles,

which often have a restricted diet, compared to wild-caught turtles, and relative abundance decreased during rehabilitation (Ahasan et al. 2017, Bloodgood et al. 2020). When compared to the fecal microbiomes of loggerheads in the North Atlantic that stranded and died due to cold stunning (Forbes et al. 2023), the trend remained consistent such that MAB turtles in the present study had lower levels of *Proteobacteria* and turtles from NC had higher levels.

Proteobacteria are commonly associated with carnivorous reptiles, such as the Chinese alligator, likely aiding in protein digestion, nitrogen metabolism, and immune modulation (Tang et al. 2019, Siddiqui et al. 2025). However, elevated *Proteobacteria* levels may serve as an early indicator of health challenges in marine species, particularly in response to environmental or anthropogenic stressors (Shin et al. 2015, Egan & Gardiner 2016, Ribas et al. 2023). The potential of *Proteobacteria* as a key phylum for monitoring sea turtle health warrants particular attention in the context of seasonal and geographic variation. For instance, if the elevated *Proteobacteria* levels observed in overwintering loggerhead turtles persist into the summer foraging months, it may indicate poor foraging success or other health challenges. This is particularly relevant throughout the Northwest Atlantic with increased marine development and habitat alteration, which are potential threats to loggerhead turtle populations (Bolten et al. 2011, Fuentes et al. 2023). *Proteobacteria* is often identified as a dominant phylum in the cloacal and fecal microbiomes of healthy sea turtles (Price et al. 2017, Ahasan et al. 2018, Samuelson et al. 2020, Scheelings et al. 2020a,b), indicating that its presence alone is not necessarily indicative of dysbiosis. Rather, interpretation of *Proteobacteria* abundance likely depends on ecological context and relative shifts within a community. The present study provides valuable baseline data on loggerhead turtle gut microbiomes prior to the development of wind farms, offering a foundation for future monitoring of potential impacts on these populations. Continued sampling and microbiome analysis can aid in the assessment of how such large-scale developments intersect with the health and ecology of loggerhead turtles in this region.

Firmicutes and *Fusobacteria* were dominant across all groups in this study. These phyla have previously been identified in the gut microbiomes of loggerheads, though relative abundances vary (Arizza et al. 2019, Biagi et al. 2019, Scheelings et al. 2020b, Filek et al. 2021, Forbes et al. 2023). Similarly, these phyla are prevalent in the fecal microbial communities of Kemp's ridley turtles (Samuelson et al. 2020). *Firmicutes* likely plays a large role in sea turtle health, consistently being identified in the herbivorous green turtles as a dominant phylum, where it is suspected to break down complex carbohydrates and plant polysaccharides to aid in digestion (Ahasan et al. 2017, 2018, Bloodgood et al. 2020). Arizza et al. (2019) suggested that the prevalence of *Firmicutes* in loggerhead turtles may be due to ingestion of seaweed and algae alongside the typical benthic organisms that constitute the majority of their diet. However, several marine carnivores, including penguins and seals, are known to have prominent levels of *Firmicutes* and *Fusobacteria* (Nelson et al. 2013, Numberger et al. 2016, Rohrer et al. 2023). Stranded green turtles, which often eat fish, had higher abundances of *Fusobacteria* compared to healthy, wild-caught turtles which feed primarily on sea grass (Ahasan et al. 2017). These observations, along with the high prevalence of *Firmicutes* and *Fusobacteria* identified in the present study, suggest that these phyla may be important to the health of the carnivorous loggerhead turtle.

Bacteroidetes was largely absent in the present study, with only 1 turtle exhibiting detectable levels. *Bacteroidetes* has been identified as a dominant phylum in previous loggerhead turtle microbiome studies, including those from the Atlantic coast (Arizza et al. 2019, Scheelings et al. 2020b, Filek et al. 2021, Forbes et al. 2023). A high *Firmicutes* to *Bacteroidetes* ratio may be a feature of carnivorous species for efficient energy harvest (Dungan & Thomas 2024). However, *Bacteroidetes* is usually considered part of the 'core' microbiome in reptiles, including carnivores such as snakes (Colston et al. 2015, Hoffbeck et al. 2023). The near absence of *Bacteroidetes* in the present study is somewhat surprising, as it contrasts with findings from other loggerhead turtle populations and might reflect differences in diet, location, or health status. Methodological factors should also be considered. In the present study, samples were rarefied to an even depth of 400 sequences per sample, and although rarefaction curves indicated that overall diversity had plateaued at this depth, limited sequencing depth may reduce the probability of detecting low-abundance taxa. Therefore, low-level representation of *Bacteroidetes* in this population cannot be excluded. More research is needed to determine if *Bacteroidetes* impacts host physiology and health of loggerhead turtles, or if previous findings have been incidental.

Interestingly, we found a high prevalence of *Epsilonbacteraeota*, especially in turtles captured in the MAB in 2023, where it was the most abundant phylum. This phylum is often found in relatively low

amounts (less than 3%) in loggerhead turtles and has not been previously identified as the most abundant phylum in any loggerhead turtle population (Arizza et al. 2019, Filek et al. 2021). Ahasan et al. (2018) found that members of *Epsilonbacteraeota* were significantly associated with stranded green turtles sampled prior to hospitalization, but not post-rehabilitation. While members of this phylum are often pathogenic in particular hosts, they have recently been found to form communities within a wide range of ectothermic reptiles and may play a generalist mutualistic role (Gilbert et al. 2019). Filek et al. (2024) suggested that loggerhead turtles, particularly their cloacal communities, may be a source of novel *Campylobacterota* (formerly *Epsilonbacteraeota*), especially of the genera *Campylobacter*, *Helicobacter*, and *Arcobacter*. We found unidentified members of those 3 genera in many of the loggerhead turtles sampled, along with members of the genus *Sulfurospirillum*. While *Epsilonbacteraeota* was the most prevalent phylum in 2023 MAB turtles, it was found in relatively low amounts in the other turtle groups, especially in NC turtles. The varying levels of *Epsilonbacteraeota* between groups suggest that prevalence of this phylum may depend on other factors. As there is high variability between each MAB turtle group, where geographic location remained consistent across sampling year, health status of individuals or temporal factors such as the availability of particular food sources are likely to contribute to the differences. More research is needed to determine the role of *Epsilonbacteraeota* in the health and physiology of loggerhead turtles, but the high prevalence found in this study of presumably healthy, wild-caught turtles suggests that it may represent a normal component of the loggerhead turtle gut microbiome rather than an indicator of dysbiosis.

Although CCL was positively correlated with alpha diversity in this study, this relationship should be interpreted cautiously, as it may reflect underlying ecological or life-history differences rather than a direct physiological effect of body size. Previous work by Filek et al. (2024) reported a negative relationship between CCL and diversity in loggerhead turtles, with the authors suggesting that smaller, and therefore younger, turtles may harbor more diverse or variable microbiomes due to differences in diet between juveniles and adults. In our data set, turtles from NC were generally smaller and exhibited lower diversity, which is likely attributable to differences in foraging behavior or resource availability rather than size alone. These contrasting findings highlight that the relationship between CCL and microbiome diversity may be influenced by ecological factors that vary across populations and over time and should be interpreted in the appropriate context.

5. CONCLUSIONS

This study provides insight into the gut microbiome of loggerhead sea turtles in the Northwest Atlantic, highlighting associations between microbial composition and factors such as geographic location, sampling year, and turtle size. Cloacal lavages offer a minimally invasive approach that may capture a broader representation of GI microbiota than cloacal swabs, though variability in fecal content must be considered in interpretations. Significant shifts in microbiome composition were observed across years and locations, perhaps influenced by foraging activity among other factors. Turtles from NC exhibited a higher relative abundance of *Proteobacteria*, which may be consistent with reduced feeding due to overwintering. In contrast, MAB turtles sampled later in the season exhibited increased alpha diversity and lower *Proteobacteria* levels, perhaps reflecting greater foraging activity or prey resources. The absence of *Bacteroidetes* and presence of *Epsilonbacteraeota* is noteworthy and warrants further investigation into the health-related significance of these phyla. These findings establish an important baseline for loggerhead turtles in the Northwest Atlantic and highlight the potential of gut microbiome monitoring as a tool for conservation and health assessments. Given ongoing environmental changes and anthropogenic development in the region, continued sampling can provide useful information for assessing impacts on population health, especially in parameters that may not be identifiable through blood chemistry alone (Scheelings et al. 2020c). Future research integrating microbiome data with traditional health metrics will enhance our understanding of how microbial communities influence loggerhead turtle physiology and support conservation efforts for this Vulnerable species.

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