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Analysis of the fecal microbiome in Kemp's ridley sea turtles *Lepidochelys kempii* undergoing rehabilitation

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ABSTRACT: The impact of the intestinal and fecal microbiome on animal health has received considerable attention in recent years and has direct implications for the veterinary and wildlife rehabilitation fields. To examine the effects of rehabilitation on the microbiome in Kemp's ridley sea turtles *Lepidochelys kempii*, fecal samples from 30 incidentally captured juveniles were collected during rehabilitation. Samples were analyzed to determine alpha- (α) and beta- (β) diversity as well as the taxonomic abundance of the fecal microbiota during rehabilitation and in response to treatment with antibiotics. The fecal microbial communities of animals housed in rehabilitation for a 'short-term' stay (samples collected 0–9 d post-capture) were compared with 'long-term' (samples collected 10+ d post-capture) and 'treated' groups (samples collected from turtles that had received antibiotic medication). Results of this study indicate that the most dominant phylum in fecal samples was *Bacteroidetes* (relative abundance, 45.44 ± 5.92% [SD]), followed by *Firmicutes* (26.62 ± 1.58%), *Fusobacteria* (19.49 ± 9.07%), and *Proteobacteria* (7.39 ± 1.84%). Similarly, at the family level, *Fusobacteriaceae* (28.36 ± 17.75%), *Tannerellaceae* (15.41 ± 10.50%), *Bacteroidaceae* (14.58 ± 8.48%), and *Ruminococcaceae* (11.49 ± 3.47%) were the most abundant. Our results indicated that both antibiotic-treated and long-term rehabilitated turtles demonstrated a significant decrease in β-diversity when compared to short-term rehabilitated turtles. Our results likewise showed that the length of time turtles spent in rehabilitation was negatively correlated with α- and β-diversity. This study demonstrates the importance of a judicious use of antibiotics during the rehabilitation process and emphasizes the importance of limiting the length of hospital stays for sick and injured sea turtles as much as possible.

KEY WORDS: Fecal microbial communities · Kemp's ridley · *Lepidochelys kempii* · Gut microbiome · Bacterial diversity · Mississippi Sound

1. INTRODUCTION

The relationship between the microbiome and overall organism health has received increased attention in recent years (Carding et al. 2015). Previous research has linked microbial diversity in the gut microbiome to positive health outcomes in several species, including amphibians (Bletz et al. 2013), fish (Ghanbari et al. 2015, Tarnecki et al. 2017), reptiles (Ahasan et al. 2017, Colston 2017), nonhuman pri-

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mates (Barelli et al. 2015, Barbian et al. 2018), and humans (Gilbert et al. 2018). The microbiome impacts development, immune response, reproduction, digestion efficiency, and overall survival (Fraune & Bosch 2010, Colston & Jackson 2016, Price et al. 2017) through its interactions with various organ systems, including respiratory (Samuelson et al. 2015), digestive (Abreu & Peek 2014), and nervous (Lyte 2013).

Kemp's ridley sea turtle *Lepidochelys kempii* is among the most critically endangered sea turtles in the world (Wibbels & Bevan 2019). Research on this species has been primarily limited to data obtained during annual arribada nesting events, which predominantly occur in Mexico and Texas (Hildebrand 1963, Marquez 1994, Shaver & Rubio 2013), and thus health data and population parameters have been informed primarily from nesting females and hatchlings. As a consequence, data regarding juvenile sea turtles are underrepresented in the literature. Opportunistic research during the rehabilitation of incidentally captured juvenile Kemp's ridley sea turtles therefore provides a unique perspective into the life history of this species (Coleman et al. 2016).

Due to the oviparous nature of the life cycle of Kemp's ridleys, nesting females do not invest energy in their offspring after depositing their eggs. However, there is evidence that eggs may be influenced by the maternal microbiome during shell development (Craven et al. 2007, Al-Bahry et al. 2009). Hatchling Kemp's ridleys navigate to surface-pelagic *Sargassum* communities, where they reside for their colloquially termed 'lost years' (Witherington et al. 2012). They remain in these ecosystems until they have reached the juvenile life stage (17–27.9 cm straight carapace length [SCL]). Opportunistically collected fecal and esophageal samples from juveniles indicate that they primarily subsist on small marine organisms, such as hydroids and portunid crabs (Witherington et al. 2012). However, as they age (SLC 20–39.9 cm), juveniles navigate to nearshore habitats (Coleman et al. 2017) where they consume blue crabs *Callinectes sapidus* and spider crabs (*Libinia* spp.; Shaver 1991, Seney & Musick 2005). Because of the complete lack of maternal care post-hatching, hatchlings rely heavily on these early stages of foraging to foster a healthy gut microbiome (Price et al. 2017) that will facilitate the turtles' transition to the various crab species that they consume as juveniles and adults (Shaver 1991, Burke et al. 1994, Seney & Musick 2005, Witzell & Schmid 2005).

Due to the broad-ranging health implications of the microbiome, it is imperative that research focuses not only on its composition in healthy sea turtles, but also on how rehabilitation and veterinary treatments, including the administration of antibiotics, impact microbial communities. This is particularly true considering that gastrointestinal issues rank as a top contributor to sea turtle strandings (Flint et al. 2010). Ahasan et al. (2018) reported that green sea turtles *Chelonia mydas* presented with very similar bacterial communities following rehabilitation, regardless of their microbial compositions at intake. Similarly, stranded green sea turtles presented with a lower bacterial diversity than wild-caught turtles (Ahasan et al. 2017). This decreased diversity in stranded turtles may be due to the introduction of external bacteria via ingestion of fishing gear (Orós et al. 2004) and marine debris, factors which impact nutrient absorption in sea turtles (McCauley & Bjorndal 1999). These findings underscore the importance of the hospital environment in influencing changes in the sea turtle microbiome.

The majority of research on sea turtle microbiomes has been strongly focused on green turtle cloacal (Ahasan et al. 2017, Price et al. 2017) and fecal (Campos et al. 2018) microbiomes and the intestinal microbiome of loggerhead sea turtles *Caretta caretta* (Abdelrhman et al. 2016). In loggerheads, the dominant microbiota found in fecal samples were *Fimicutes, Bacteroidetes*, and *Proteobacteria* — regardless of age class and overall health condition (Abdelrhman et al. 2016, Arizza et al. 2019). The prevalence of *Fusobacteria*, which is also prevalent in marine mammal fecal microbiomes, is likely due to a diet rich in fish (Biagi et al. 2019). The loggerhead microbiome may be impacted by hospitalization (Abdelrhman et al. 2016), although Biagi et al. (2019) suggested that the sea turtle microbiome is relatively stable throughout short-term hospital stays.

Green sea turtles differ greatly from Kemp's ridleys in both diet and habitat selection. Thus, it is likely that substantial differences exist in the composition and role of the microbiome between these species. Here we aimed to (1) summarize the fecal microbiome of immature Kemp's ridley sea turtles and (2) outline the impacts of rehabilitation on the microbiome with and without the use of antibiotics.

2. MATERIALS AND METHODS

2.1. Study site

The Institute for Marine Mammal Studies (IMMS) in Gulfport, Mississippi (USA), rescues and rehabili-

tates sick and injured sea turtles along the Mississippi Gulf Coast, which borders the Mississippi Sound (MSS). The MSS is a 1–7 m deep embayment (Eleuterius 1978) spanning 2130 km^2 and separated from the greater Gulf of Mexico by barrier islands (Cat, Ship, Horn, Petit Bois, and Dauphin Islands; Kjerfve 1986). The MSS harbors shallow seagrass beds (Moncreiff et al. 1992) and a thriving blue crab fishery (Rakocinski et al. 2003), making the MSS suitable habitat for juvenile sea turtles.

2.2. Sample selection

The majority of the turtles admitted to the hospital for rehabilitation were juvenile Kemp's ridleys, which had been incidentally captured by recreational anglers on local fishing piers but were otherwise considered to be healthy individuals (Coleman et al. 2016). This presented a unique opportunity to examine the behavior, biology, and ecology of this Critically Endangered species (Wibbels & Bevan 2019), particularly the understudied juvenile age class, as determined by SCL.

Fecal samples were collected from 30 incidentally captured juvenile Kemp's ridley sea turtles housed at IMMS for the purposes of rehabilitation. The turtles sampled in this study were considered to be free of confounding illnesses. Subjects were treated and/or held for observation due to oral, tracheal, esophageal, and/or external injuries that were incurred as a result of incidental capture. Thus, the cause of their hospitalization was not likely to have an impact on their intestinal or fecal microbiota. Subjects were sorted into 3 groups at the time of sampling: 'short-term' (collected 0–9 d post-capture), 'long-term' (collected 10+ d post-capture), and 'antibiotic-treated' (collected 10+ d post-capture from sea turtles that had been treated with the antibiotic ceftazidime while in rehabilitation; Table 1). Subjects were categorized opportunistically, as a result of the veterinarians' existing orders. The veterinary treatment, husbandry, and eventual release of the turtles was not impacted in any way by this study.

Fecal samples were collected at the completion of the turtles' respective rehabilitation periods, immediately preceding their release. The samples were collected directly from the rehabilitation pools, where turtles were housed individually. Rehabilitation pools were approximately 1 × 0.9 × 1 m in size and were filled approximately 36″ (0.91 m) deep at the time of collection. Saltwater was filtered prior to placement in the pools and was changed daily. Pools were disinfected between fills using dilute chlorhexidine, which was allowed to sit in the drained pool for 15 min prior to thoroughly rinsing and refilling the pool.

Antibiotic-treated turtles received intramuscular ceftazidime, which is a third-generation cephalosporin known to be effective against Gram-positive and Gram-negative aerobic bacteria (Stamper et al. 1999). However, in reptiles, it is particularly effective against *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Richards & Brogden 1985), as well as *Vibrio* spp. and *Aeromonas* spp. (Stamper et al. 1999). Intramuscular injections of ceftazidime, as were administered in this case, have been demonstrated to be effectively absorbed and distributed throughout the body in loggerhead (Stamper et al. 1999) and Kemp's ridley sea turtles (Innis et al. 2012). For subjects included in the present study, ceftazidime was prescribed as a preventative measure for cases in which more severe injuries and/or injuries deemed by the veterinarian likely to become infected were observed at intake. Subjects suspected to have active infections, and/or subjects whose treatment included additional or alternative antibiotic treatments, were excluded from this study.

Turtles were released into the Mississippi Sound once pronounced releasable by the attending veterinarian, and the environmental conditions were deemed favorable.

2.3. DNA extraction

Once all fecal samples had been collected and preserved in RNAlater®, they were placed on ice and shipped to the Southeastern Cooperative Fish Parasite and Disease Laboratory (Auburn University, Alabama, USA) for DNA extraction. Upon arrival, samples were stored at −80°C until processing.

Selected samples were later transferred to −20°C for temporary storage and partial thawing of samples frozen in RNAlater®. Prior to DNA extraction, samples were removed from the −20°C freezer, immediately placed on ice, and allowed to thaw slowly. In an effort to reduce potential problems in downstream microbial analyses from the excess salts found in RNAlater®, technicians first gently washed the fecal samples in ice cold, sterile phosphate-buffered solution 3 times. Washed fecal contents were homogenized using a handheld homogenizer and then transferred into sterile, pre-weighed, 2.0 ml Eppendorf microcentrifuge tubes until a target weight of 160–180 mg (or the highest available volume if less) per sample was reached.

Table 1. Details of juvenile Kemp's ridley turtles sampled during rehabilitation, and treatment conditions (short: short-term rehabilitation, long: long-term rehabilitation, antibiotic treated: treated with ceftazidime while in rehabilitation). SCL: straight carapace length; CCL: curved carapace length. Length measurements were made notch to notch; length and weight data were taken at the time of release. Dates are given as mo/d/yr

Sample ID	SCL (cm)	CCL (cm)	Weight (kg)	Release date	Days in rehabilitation at time of sampling	Condition
N1	30.9	32.5	4.3	8/27/14	5	Short
N2	28.2	29.3	3.1	9/4/14	2	Short
N3	31.3	32.2	4.1	9/4/14	0	Short
N4	31.5	32.7	4.2	9/4/14	3	Short
N5	31.2	33.0	4.2	9/4/14	2	Short
N6	28.4	29.6	3.3	10/31/14	0	Short
N7	39.1	41.6	8.6	10/1/14	9	Short
N8	32.0	33.3	5.3	10/9/14	2	Short
N9	28.7	30.4	3.2	10/17/14	0	Short
N10	26.5	27.7	3.2	10/31/14	2	Short
L1	29.5	31.5	3.7	7/25/14	11	Long
L2	37.3	39.2	6.6	8/15/14	19	Long
L3	31.8	33.1	4.5	9/19/14	16	Long
L4	26.9	28.5	3.0	10/9/14	46	Long
L5	30.9	32.5	4.3	8/27/14	12	Long
L6	30.9	32.1	4.2	10/31/14	36	Long
L7	30.7	32.0	3.9	9/19/14	13	Long
L8	32.9	34.7	5.6	9/25/14	16	Long
L9	32.4	34.2	4.7	10/1/14	16	Long
L10	39.1	41.6	8.6	10/1/14	17	Long
T1	31.0	32.7	4.1	10/1/14	78	Antibiotic treated
T2	30.3	31.6	3.9	9/4/14	43	Antibiotic treated
T3	32.0	33.7	4.5	9/19/14	50	Antibiotic treated
T4	29.7	31.0	3.8	10/17/14	88	Antibiotic treated
T5	34.9	36.7	5.1	10/17/14	30	Antibiotic treated
T6	38.2	40.0	7.0	10/17/14	51	Antibiotic treated
T7	28.4	29.6	3.3	10/31/14	13	Antibiotic treated
T8	33.7	35.1	5.7	2/2/15	41	Antibiotic treated
T9	44.9	47.0	10.9	11/5/14	10	Antibiotic treated
T10	33.7	35.1	5.7	2/2/15	21	Antibiotic treated

All DNA extractions were performed using the QIAmp® DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions, with only minor changes. Modifications to the 'Isolation of DNA from Stool for Pathogen Detection' protocol included the following: (1) the addition of ice cold, 100% ethanol to the lysate during the binding step; (2) the use of warm Buffer AE during the elution step; (3) the addition of 50 µl of warmed Buffer AE directly to the spin column during elution; and (4) the extension of the final incubation period to 2 min at room temperature prior to elution of the DNA via centrifuging. Total DNA concentrations were then quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies), and all samples were checked for amplification of 16S DNA prior to sending samples off for sequencing.

2.4. DNA sequencing of the 16S rRNA gene

In total, 30 samples were submitted to MR DNA® (www.mrdnalab.com, Shallowater, Texas, USA) for PCR amplification and next-generation sequencing. Universal bacterial primers 515 F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) with a barcode on the forward primer were used to target the 16S rRNA gene V4 variable region. The HotStarTaq Plus Master Mix Kit (Qiagen) was used to run all samples under the following PCR conditions: an initial denaturation step for 3 min at 94°C followed by 28 cycles of 94°C for 30 s (denaturing), 53°C for 40 s (annealing), and 72°C for 1 min (extension) before performing a final elongation step for 5 min at 72°C. Following amplification, PCR products for all samples were run through a 2% agarose gel to verify successful amplification and relative band intensity of the target DNA. Multiple samples were pooled together and purified using calibrated Ampure XP beads to prepare the Illumina DNA library prior to sequencing.

2.5. Sequence curation and analysis

Raw sequence data were processed using R and the R packages: 'DADA2' v1.1.5, 'DECIPHER' v2.12.0, 'Phyloseq' v1.16.2, 'DESeq2' v1.20.0, and 'vegan' v2.3-5 (Anders & Huber 2010, McMurdie & Holmes 2012, 2015, Callahan et al. 2016, Murali et al. 2018, Oksanen 2019). Sequences were truncated to 250 bp, denoised, chimera-filtered, and clustered into sequence variants using 'DADA2.' Operational taxonomic units (OTUs) were generated in 'DADA2' by taxonomic classification of sequence variants using 'DECIPHER IDTAXA' and the 'SILVA' reference database v132. Alpha- (α) and beta- (β) diversities, as well as taxonomic community assessments were analyzed via the R package 'Phyloseq.' The number of unique sequence variants in a sample (α-diversity) was calculated using the 'estimate richness' function in Phyloseq. Bray-Curtis dissimilarity (β-diversity) was calculated using the 'vegdist' function in 'vegan' with raw OTU counts. Differen-

tially abundant OTUs were determined via 'DESeq2.' All graphics were generated using 'ggplot2' (Wickham 2016).

2.6. Statistical analysis

We modeled α-diversity using generalized linear models (GLMs) available within R (version 3.6.1). Model variables included rank time in rehabilitation, the presence of antibiotic treatment, and sequencing depth. Rank time in rehabilitation was calculated by taking the square root of the days in the rehabilitation setting to ensure a normal distribution of days. Nonmetric multidimensional scaling (NMDS) was performed on sample-wise Bray-Curtis dissimilarity distances to assess β-diversity. Significant effects of independent model covariates on NMDS clustering were inferred via permutational multivariate analysis of variance (PERMANOVA) using distance matrices within R. Pairwise comparisons were performed with corrections for multiple comparisons via false discovery rate (FDR) (Benjamini & Hochberg 1995). A p-value <0.05 and an FDR q-value <0.1 were considered statistically significant.

3. RESULTS

3.1. Time in rehabilitation and antibiotic treatment alters the diversity of fecal microbial communities

Fecal samples from 30 incidentally captured juvenile Kemp's ridley sea turtles were sequenced and 654 905 raw reads were obtained. Over 80% (536 622 total reads) of the raw reads were maintained following filtering, denoising, merging, and removing chimeras (Table 2). Utilizing the 'DADA2' pipeline, we identified 3327 unique OTUs and classified them using 'DECIPHER' at a 97% sequence similarity threshold against the SILVA reference database. Fecal samples contained between 65 and 139 OTUs, and were classified into 17 phyla, 25 classes, 33 orders, and 47 families.

Terminal microbial community structure was analyzed from fecal samples collected during rehabilitation. Neither the time a turtle spent in rehabilitation nor whether it received antibiotics resulted in marked changes to its microbial α-diversity, as measured by Chao1, Simpson, InvSimpson, Shannon, Fisher, or abundance-based coverage estimator (ACE) diversity measurements (Table 3). While not significant, the number of observed bacterial OTUs was reduced in long-term rehabilitation and antibiotic-treated turtles when compared to turtles that underwent short-term rehabilitation (Fig. 1A).

β-diversity of the microbial communities from short-term, long-term, and antibiotic-treated turtles was determined using sample-wise Bray-Curtis dissimilarity distances, and significant effects of independent model covariates on NMDS clustering were inferred via PERMANOVA using distance matrices. NMDS plots show that significant differences in β-diversity existed between long-term and short-term ($q = 0.0021$) turtles, as well as between short-term and

Table 2. Summary of the numbers of reads of fecal microbial communities sampled from 30 juvenile Kemp's ridley turtles. OTU: operational taxonomic unit

Sample ID	Input reads	Filtered reads	Denoised forward reads	Denoised reverse reads	Merged reads	Non-chimera reads	Number of OTUs
L1	21168	19050	18834	18786	18282	17553	84
L2	23899	21200	20746	20707	19726	19151	126
L3	24531	21883	21502	21462	20622	20374	118
L4	19197	17148	16880	16853	16451	16063	100
L5	21458	19486	19093	19109	18292	17710	109
L6	16603	14916	14662	14647	14129	14065	100
L7	20104	18185	17786	17822	17241	17142	136
L8	27935	24947	24635	24512	23322	22793	105
L9	25787	23168	22765	22751	22161	21926	110
L10	19685	17612	17318	17303	16692	16574	120
N1	24574	22263	21804	21769	20502	19973	139
N2	27738	24840	24377	24352	23242	22961	171
N3	23965	21401	21047	21043	19816	19382	110
N4	21457	19123	18747	18688	17721	17095	119
N5	20258	18121	17856	17813	17002	16868	96
N6	14636	12943	12662	12591	12097	12097	134
N7	27872	24818	24323	24373	23037	21541	111
N8	24370	21438	21084	21115	20165	18967	106
N9	15059	13446	13179	13140	12533	12353	106
N10	15037	13278	13005	13036	12560	12211	97
T1	22972	20641	20296	20252	19450	19261	92
T2	23829	21005	20767	20741	19617	19098	65
T3	23492	20946	20598	20597	19330	18943	87
T4	20755	18389	18057	18045	17491	17414	122
T5	28621	25422	24971	24955	23585	22921	124
T6	19635	17440	17111	17075	16548	16548	171
T7	20418	18215	17890	17860	17197	17132	130
T8	25877	23033	22591	22548	21166	20030	94
T9	10156	8910	8746	8763	8462	8462	70
T10	23817	21605	21332	21328	20626	20014	75

antibiotic-treated (q = 0.0025) individuals, as determined by PERMANOVA via 'vegan' (Fig. 1B). However, no significant difference (q = 0.1586) was observed between long-term and antibiotic-treated turtles (Fig. 1B).

3.2. Time in rehabilitation and antibiotic treatment changes the abundance of specific bacterial taxa

We assessed the differences in taxonomic composition between short-term rehabilitation, long-term rehabilitation, and antibiotic-treated turtles. Specifically, the most dominant phylum in fecal samples was *Bacteroidetes*, with an average (±SD) relative abundance of 45.44 ± 5.92%, followed by *Firmicutes* (26.62 ± 1.58%), *Fusobacteria* (19.49 ± 9.07%), and *Proteobacteria* (7.39 ± 1.84%). Less represented were *Euryarchaeota, Actinobacteria*, *Spirochaetes, Lentisphaerae*, *Epsilonbacteraeota*, and *Verrucomicrobia*, (ranging from 0.311–0.096% relative abundance, respectively). Similarly, at the family level, *Fusobacteriaceae* (28.36 ± 17.75%), *Tannerellaceae* (15.41 ± 10.50%), *Bacteroidaceae* (14.58 ± 8.48%), and *Ruminococcaceae* (11.49 ± 3.47%) were the most abundant. Fig. 2A shows the breakdown of the relative abundance of taxa at the genus level in short-term rehabilitation, long-term rehabilitation, and antibiotic-treated turtles.

Comparisons between the microbial communities in each group demonstrated significant changes in the relative abundance of specific OTUs as determined by 'DESeq2.' Specifically, 8 OTUs were more abundant in long-term rehabilitation turtles than short-term turtles, while 8 OTUs were more abundant in short-term rehabilitation turtles when compared to long-term turtles (Fig. 2B). Similarly, 8 OTUs were more abundant in antibiotic-treated turtles compared to short-term turtles, while 7 OTUs were more abundant in short-term rehabilitation turtles than antibiotic-treated turtles (Fig. 2C). Finally, 1 OTU was more abundant in long-term rehabilitation turtles when compared to antibiotic-treated turtles, and 5 OTUs were more abundant in antibiotic-treated turtles than long-term rehabilitation turtles (Fig. 2D).

Table 3. Summary of α-diversity indices of fecal microbial communities sampled from 30 juvenile Kemp's ridley turtles. OTU: operational taxonomic unit; ACE: abundance-based coverage estimator

Sample ID	Observed OTUs	Chao1	Simpson	Inv-Simpson	Shannon	Fisher	ACE
L1	84	84	0.915	11.767	3.070	11.451	84
L2	126	126	0.933	15.006	3.593	18.088	126
L3	118	118	0.969	32.005	3.832	16.586	118
L4	100	100	0.926	13.434	3.376	14.224	100
L5	109	109	0.931	14.419	3.375	15.475	109
L6	100	100	0.918	12.188	3.312	14.545	100
L7	136	136	0.977	42.920	4.121	20.157	136
L8	105	105	0.928	13.814	3.323	14.228	105
L9	110	110	0.956	22.487	3.560	15.108	110
L10	120	120	0.947	18.725	3.651	17.508	120
N1	139	139	0.941	16.821	3.539	20.144	139
N2	171	171	0.949	19.771	3.882	25.070	171
N3	110	110	0.883	8.574	3.044	15.411	110
N4	119	119	0.945	18.063	3.468	17.247	119
N5	96	96	0.897	9.673	3.030	13.456	96
N6	134	134	0.964	28.155	3.986	21.090	134
N7	111	111	0.929	14.101	3.327	15.311	111
N8	106	106	0.852	6.744	2.771	14.813	106
N9	106	106	0.783	4.618	2.595	15.928	106
N10	97	97	0.878	8.206	3.151	14.380	97
T1	92	92	0.933	14.849	3.246	12.538	92
T2	65	65	0.880	8.314	2.669	8.411	65
T3	87	87	0.843	6.386	2.784	11.784	87
T4	122	122	0.964	27.924	3.860	17.701	122
T5	124	124	0.922	12.879	3.271	17.238	124
T6	171	171	0.950	20.083	3.820	26.570	171
T7	130	130	0.958	23.689	3.723	19.120	130
T8	94	94	0.956	22.549	3.523	12.775	94
T9	70	70	0.794	4.863	2.173	10.451	70
T10	75	75	0.806	5.148	2.313	9.846	75

3.3. Days in rehabilitation correlate with loss of α-diversity and changes in β-diversity

We determined that the number of days that a turtle spent in rehabilitation had a negative association with α-diversity (rho = −0.2417 p = 0.198), as determined by multiple general linear regression (Fig. 3A). Model covariables included antibiotic treatment and sequencing depth. β-diversity was also significantly associated with days in rehabilitation (p = 0.0001, PERMANOVA) (Fig. 3B).

4. DISCUSSION

Both the structure of microbial communities and the taxonomic abundance of the fecal microbiota were sig-

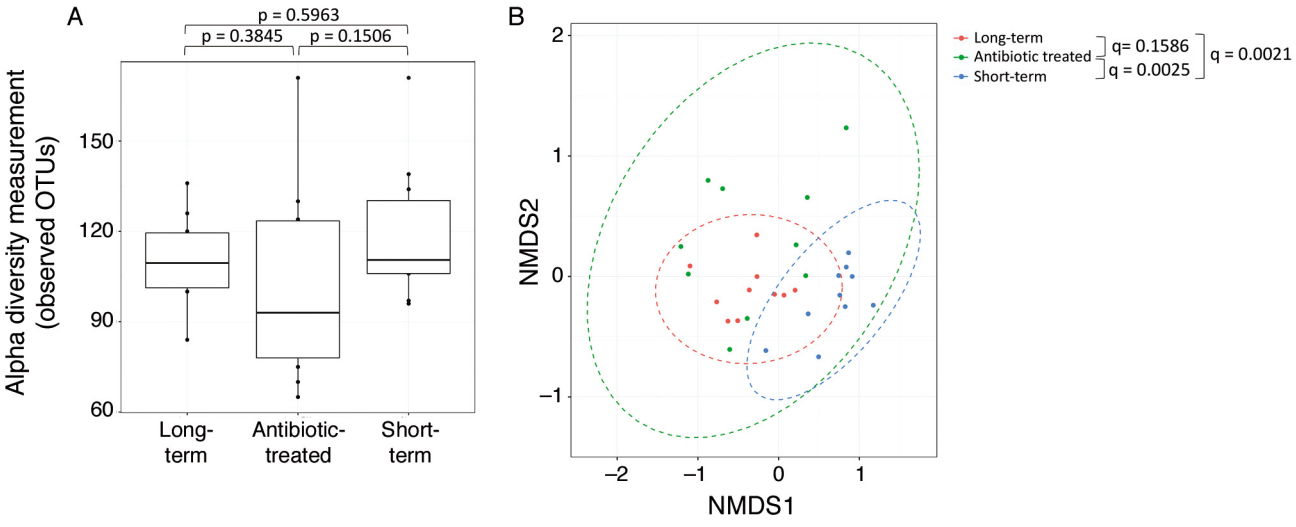

Fig. 1. (A) α-diversity (determined by multiple general linear regression) and (B) β-diversity (determined by PERMANOVA in R) of fecal microbial communities from Kemp's ridley sea turtles in rehabilitation (short-term, long-term, and antibiotic-treated). In (B), pairwise comparisons were performed with corrections for multiple comparisons via false discovery rate (FDR), and significant differences (FDR q-value <0.05) were observed between long-term and short-term, as well as between short-term and antibiotic-treated animals

nificantly affected by the length of stay in rehabilitation, as well as the use of antibiotics. α-diversity and β-diversity metrics were also correlated with the length of stay in rehabilitation, when accounting for antibiotic treatment. As the relationship between the fecal microbiome and its impact on an animal's overall health is poorly understood, additional research is required to fully understand the implications of these changes on an individual's long-term health outcomes.

The characterization of the fecal microbiome for sea turtles worldwide is an area of growing interest. To our knowledge, our study is the first to examine the fecal microbiome of Kemp's ridley sea turtles and the influence of rehabilitation on the microbiome of this species. Our results demonstrate that the most dominant phylum in fecal samples from Kemp's ridley sea turtles were *Bacteroidetes, Firmicutes*, *Fusobacteria*, and *Proteobacteria*. This coincides with findings from loggerheads (Abdelrhman et al. 2016, Arizza et al. 2019), which may be due at least in part to an overlap in dietary preferences and habitat selection between the 2 species. In addition, the fecal microbiota was significantly affected by the length of stay in rehabilitation, as well as by the use of antibiotics. Specifically, we observed changes in several diversity metrics, as well as a significant reduction in the abundance of *Bilophila, Butyricomonas, Eubacterium, Macellibacteroides Parabacteroides, Paraeggerthella, Tyzzerella*, and *Vibrio* in long-term rehabilitated turtles when compared to short-term rehabilitated turtles. These results are similar to results seen in green sea turtles, where *Bilophila* were enriched in pre-rehabilitation turtles compared to post-hospitalized turtles. Further, our results revealed that long-term or treated turtles exhibited a significant increase in *Moritella* and *Photobacterium* (*Proteobacteria*) compared to short-term turtles.

We found that turtles undergoing long-term rehabilitative care and turtles treated with antibiotics demonstrated a significant decrease in the abundance of several genera that could have potential health implications. For example, *Butyricomonas* and *Eubacterium* produce butyrate, a short-chain fatty acid essential for intestinal health (Amato et al. 2013). Additionally, increased levels of *Bilophila* in short-term turtles could indicate that these organisms are important for normal gut physiology and nutrition, as suggested for green turtles (Ahasan et al. 2018). Converse to what was observed in green turtles (Ahasan et al. 2017), we found the genus *Vibrio* to be enriched in short-term rehabilitated turtles compared to long-term or treated turtles. However, *Vibrio* could have varying health implications depending upon the species of bacteria. For example, *V. harveyi, V. owensii,* and *V. parahaemolyticus* can indicate an opportunistic infection (Ahasan et al. 2018), while other species common to the microbiome of marine crustaceans and mollusks (*V. xuii* and *V. pomeroyi*, for example) have been found to be

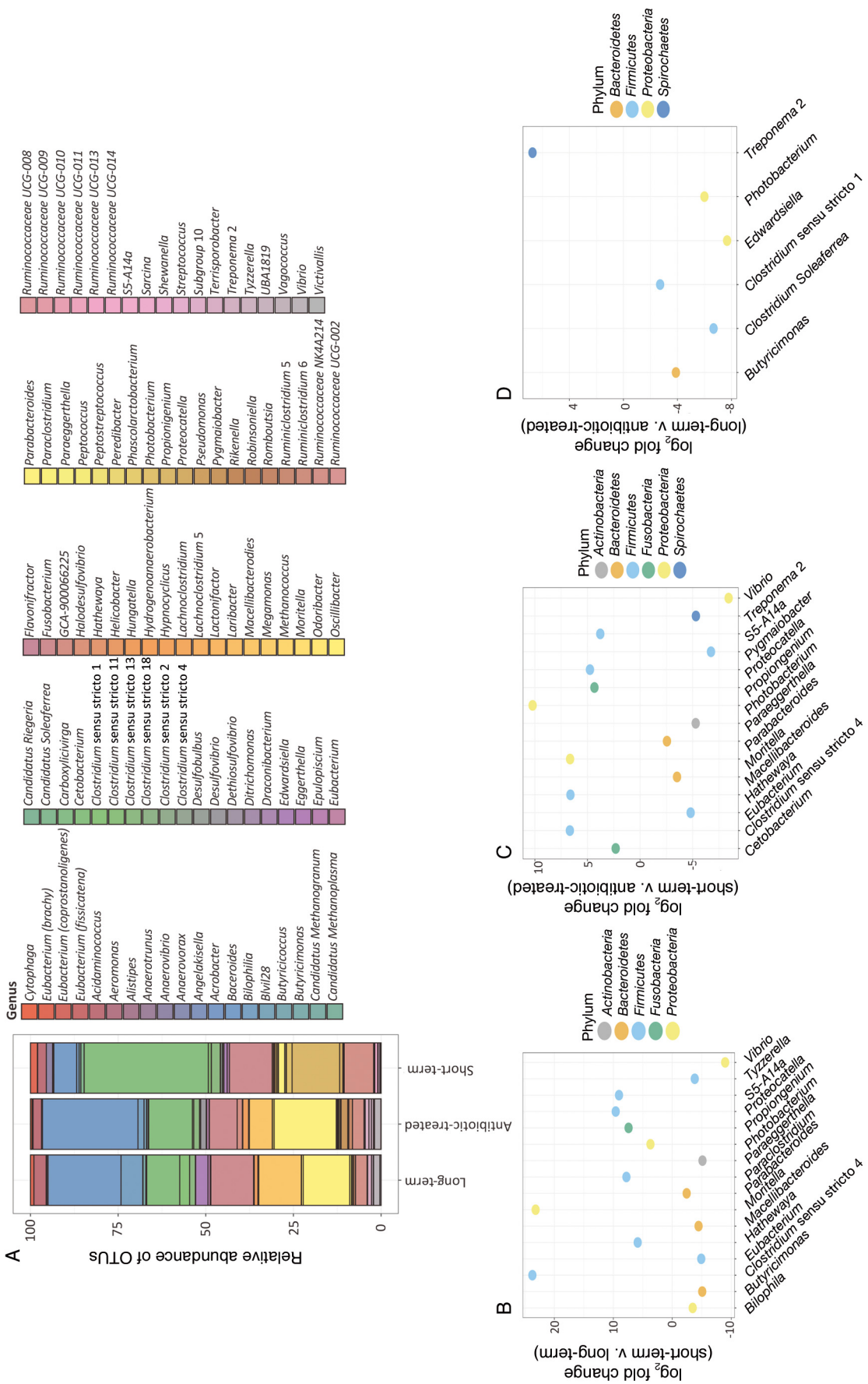

Fig. 2. (A) Relative abundances of bacterial taxa in short-term rehabilitation, long-term rehabilitation, and antibiotic-treated Kemp's ridley turtles. (B–D) Relative abundances of specific operational taxonomic units (OTUs) in the fecal microbiota. In (B), positive (negative) changes indicate that the genus is more abundant in long-term (short-term) rehabilitation animals. In (C), positive (negative) changes indicate that the genus is more abundant in the antibiotic-treated (short-term rehabilitation) animals. In (D), positive (negative) changes indicate that the genus is more abundant in the long-term rehabilitation (antibiotic-treated) animals

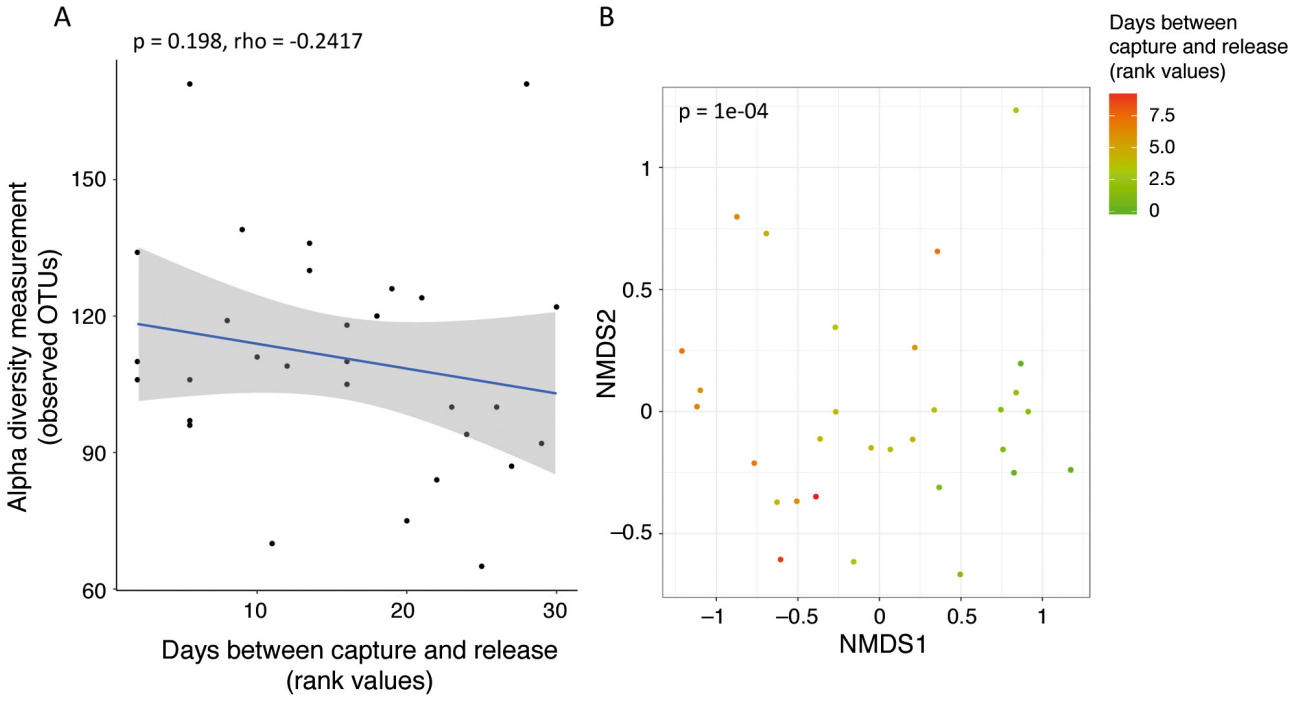

Fig. 3. Time that Kemp's ridley turtles spent in rehabilitation was associated with both (A) loss of bacterial α-diversity determined by multiple general linear regression (OTU: operational taxonomic unit) and (B) changes in β-diversity determined by PERMANOVA within R. Model covariables included antibiotic treatment and sequencing depth. The blue line represents the best-fit regression line, and the grey shaded area is the 95% confidence interval. NMDS: nonmetric multidimensional scaling

either non-virulent, or have a very low virulence in animal models (see Romalde et al. 2014 for a review). In addition, *Vibrio* may play a different biological role in Kemp's ridleys than it does in green turtles, as so little is known about the direct linkages between the microbiome and animal health outcomes. Finally, we found that *Proteobacteria* abundance increased in long-term and treated turtles. Interestingly, *Proteobacteria* have been suggested to be indicators of dysbiosis in sea turtles (Arizza et al. 2019). In the present study, we did observe increases in several *Proteobacteria* members, which would support previous associations with dysbiosis. However, we also found *Proteobacteria* members which were enriched in short-term turtles, suggesting that certain *Proteobacteria* may be markers of dysbiosis in sea turtles.

These findings, together with the overall changes in α- and β-diversity, reinforce the understanding that antibiotics should be used sparingly and only when necessary for the health of the animal. Additionally, the reduction in microbial diversity observed in long-term rehabilitation suggests that rehabilitators should attempt to limit the length of time animals spend in rehabilitation as much as possible. Of course, this is not possible in the case of severe injury or illness which sometimes warrants long-term hospitalization. However, further work is needed to understand if supplementation with probiotics, or exposure to locally caught seafood as an exclusive diet, may assist in maintaining normal microbial diversity throughout rehabilitation.

Future studies should also examine the Kemp's ridley microbiome at the species level and consider the role sex differences may play. However, the present study represents an important first step in understanding the role of the sea turtle microbiome and how it can ensure and improve the species' overall health as they undergo rehabilitation.

Work by Biagi et al. (2019) suggests a strong link between diet and the fecal microbiome, which did not appear to be influenced by the length of hospital stay or the environment (i.e. water in the rehabilitation pools etc.). This suggests that if the rehabilitation facility was capable of including fresh, locally-sourced prey species in the turtles' diet, they would be more likely to maintain a healthy and diverse gut microbiome. Future research should examine the role of diet on the microbiota, including the role of freezing fish and other practical considerations regarding diet preparation.

Additionally, the role of the microbiome must be considered in species preservation plans that involve managed zoological or rehabilitative care, given that dysbiosis, a divergence from the normal microbial community, is strongly associated with the development of disease. Thus, caregivers and veterinarians should consider the addition of pre- and probiotics to daily care regimens, as well as increasing diet diversity (West et al. 2019). Additionally, it should be noted that both habitat degradation and climate change have been demonstrated to impact the structure and function of the microbiome in a variety of species (Apprill 2017, West et al. 2019). Therefore, it is critical that evaluations of the Kemp's ridley microbiome be repeated over time as a method for monitoring long-term changes in sea turtle and environmental health.

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