

Analysis of the Stomach Tissue Microbiome within Florida Manatees, *Trichechus manatus*
latirostris

Samantha Hamontree

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Advised by: Dr. Melanie Langford

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Abstract

Identification of the microbiome within marine mammals, including sirenians has been the focus of recent research. While previous work has identified the microbiome from the lower gastrointestinal tract (fecal samples) of manatees and dugongs, we sought to examine the microbiome of the upper gastrointestinal tract, specifically the stomach of Florida manatees, *Trichechus manatus latirostris*. We obtained two stomach tissue samples (one sample from the greater curvature of the stomach and the other from the lesser curvature of the stomach) from three recently deceased Florida manatees in collaboration with the Marine Mammal Pathobiology Laboratory in St. Petersburg, FL between 2013-2016. These samples were stored at -80C until processing, when DNA was extracted from the thawed stomach tissue using the Zymobiomics DNA miniprep kit. Samples were sent for metagenomics sequencing utilizing the V3 and V4 variable regions of 16S rRNA gene at Genewiz (New Jersey, USA). As expected, several species of fermenting microorganisms were found as well as cellulose degrading microorganisms. Statistical tests were implemented to compare bacterial abundance and diversity between manatees and the two tissue sections that were sampled. Here, we are the first to report the microbiome composition inhabiting the upper gastrointestinal tract within any sirenian. While this is the first study to describe the stomach microbiome of Florida manatees, our findings will be available for future studies as a baseline for the microbiome of the upper gastrointestinal tracts of sirenians. Since the composition of the microbiome has been linked to health in other mammals, this research project may provide important information to veterinary care providers.

Introduction

The Order Sirenia is composed of two families: Dugongidae and Trichechidae (Parsons, 2013). The three species of manatees within Trichechidae that are extant include the Amazonian manatee (*Trichechus inunguis*), the West African (*Trichechus senegalensis*) and the West Indian manatee (*Trichechus manatus*). The West Indian manatees are further classified into two subspecies: the Antillean (*Trichechus manatus manatus*) and the Florida manatee (*Trichechus manatus latirostris*). While Florida manatees typically are found along the coast of Florida in brackish to marine waters, their summer range can stretch from Texas towards Rhode Islands. However, in the winter months with temperatures below 20°C, manatees will migrate to warm water refuges within Florida (Laist and Reynolds, 2005). These refuges include warm-water, natural springs and estuaries surrounding power plants (Laist and Reynolds, 2006). Sirenians are the only marine mammals that are obligate herbivores, with the diet of manatees typically including a variety of sea grass and floating aquatic vegetation (Lefebvre et al., 2001) (Parsons, 2013).

Gastrointestinal Tract

To date, all culture-independent studies of the microbiome in Sirenians have focused on the lower gastrointestinal tract microbiome, specifically fecal sampling. Suzuki et al (2019) collected fecal samples from four Antillean manatees in captivity in Japan. Through sequencing the V3-V4 region of bacterial 16S rRNA, the most dominant bacterial phyla identified was Firmicutes at 84.05% (Suzuki et al., 2019). The second most prevalent phyla was Bacteroidetes with an abundances of 8.60% (Suzuki et al., 2019). In a different study by Merson et al. (2014), the microbiome of wild Florida manatee hindguts were also evaluated using 16S rRNA gene sequencing through identifying the V3-V4 region. Firmicutes (77.3%) was the phylum with the

largest relative abundance and Bacteroidetes (19.5%) was the second largest relative abundance (Merson et al., 2014).

Samples were taken from the hindgut of over 200 wild dugongs from the Moreton Bay in Eastern Australia from both sexes and a variety of ages and from 2 captive dugongs from Sea World, Queensland, Australia (Eigeland et al., 2013). The V2-V3 region of the bacterial 16S rRNA gene was amplified for identification (Eigeland et al., 2013). The two most abundant phyla found were Firmicutes (75.6%) and Bacteroidetes (19.9%) (Eigeland et al., 2013). Fecal samples were collected from a captive female dugong in Toba Aquarium in 2000, 2004, and 2005 (Tsukinowa et al., 2008). Six bacterial phyla were identified, with majority of the sequenced DNA belonging to Firmicutes (83.1%) followed by Bacteroidetes (15%) (Tsukinowa et al., 2008). It was also observed that there was a large variation in the bacteria observed amongst samples from the same dugong (Tsukinowa et al., 2008).

While the upper gastrointestinal tract has not been previously studied in sirenians, it has been studied in other marine mammals. Forty-four recently deceased southern right whale (*Eubalena australis*) calves' gastrointestinal tracts were characterized with high-throughput sequencing as well as through culture methods. There were 108 bacterial genera found, with majority belonging to Firmicutes and Bacteroidetes (Maron et al., 2019). The upper gastrointestinal tract of Pygmy (*Kogia breviceps*) and Dwarf (*Kogia sima*) Sperm Whales revealed that the highest abundance was Firmicutes and Bacteroidetes (Erwin et al., 2017).

Other studies have viewed the microbiome of the gastrointestinal tract in herbivorous animals, to include horses and elephants. Through extracting DNA from the V1-V2 region of 16SrDNA, upper and lower gastrointestinal tracts were analyzed in 10 horses (Dougal et al., 2013). It was found that in both the upper and lower gastrointestinal tract, the predominant

phylum was Firmicutes and the next relevant phylum was Bacteroidetes (Dougal et al., 2013). Another study on feral horses using 16S rRNA found that there were four major bacterial types in the upper gastrointestinal tract (St-Pierre et al., 2012). Three of the four bacterial types belong to Firmicutes while the fourth belongs to Proteobacteria (St-Pierre et al., 2012). The lower gastrointestinal tract was studied in two elephants, a three-week-old and a six-year-old through 16S rRNA sequencing of the V3-V5 region. In the three-week-old elephant, over 50% of the bacteria identified was Bacteroidetes (Ilmberger et al., 2014). The next two most abundant phyla were Proteobacteria (32%) and Firmicutes (11%) (Ilmberger et al., 2014). In the six-year-old elephant, the two most abundant phyla were Bacteroidetes (47%) and Firmicutes (36%) (Ilmberger et al., 2014).

Specific Aim

This will be the first study of its kind to describe the bacterial component of the normal microbiome of the upper gastrointestinal tract in Sirenians. Our first specific aim is to extract bacterial DNA from stomach tissue samples of 3 Florida manatees through 16S rRNA gene sequencing. Our second specific aim is to compare the microbiome of the upper gastrointestinal tract to previous studies of the lower gastrointestinal tracts of other Sirenians. With the upper gastrointestinal tract unknown in Sirenians, it is important to expand upon the knowledge to create a clearer understanding of what is present. The composition of the upper gastrointestinal tract is important since bacterial microbiome has been previously linked to health in other mammals. Based on previous research, we hypothesize to find a high abundance of Firmicutes and Bacteroidetes in the stomach tissue of Florida manatees. We also hypothesize to see no difference between the lesser curvature and greater curvature of the stomach tissue samples due

to their proximity. Lastly, we expect to see little variation between manatees, due to all three manatees being found deceased in the wild.

Material and Methods

Sample Collection and Storage

Stomach tissue samples were collected from three recently deceased Florida manatees in collaboration with Marine Mammal Pathobiology Lab in St. Petersburg, Florida in 2013, 2014, and 2016. Detailed information on the manatees is shown in Table 1. Lesser curvature and greater curvature stomach tissue samples were taken from each manatee. The samples were then transported to Florida Southern College in Lakeland, Florida where the samples were stored in -80°C in 20% glycerol prior to extraction.

Table 1: Identifying information for the three manatees used in this study to include field ID number, sex, date collected, location collected and the probable cause of death

Manatee Tag	Sex	Date Collected	Location Collected	Probable Cause of Death
LPZ 103099 (Manatee 1)	Female	07/29/2013	Clearwater, FL; approx.. 100m NNE of intersection of Sunset Dr. and Venetian Dr.	Watercraft collision
MNE14001 (Manatee 2)	Male	1/15/2014	Georgetown, FL; NE side of Hog Island near boat slips. Approx.. 2km W of North Point Dr.	Cold stress
MEC 16019 (Manatee 3)	Female	2/15/2016	Port St. John, FL; near the FPL warm water discharge	Cold stress

DNA extraction, PCR amplification and sequencing

The tissue samples were thawed and a cube of roughly 1cm by 1cm were removed from each tissue sample. This cube then had Qiagen proteinase K and Qiagen ATL added to it and was stored in 55°C water bath overnight to dissolve the tissue. Zymobiomics DNA miniprep kit methods were followed in order to utilized to extract the microbial DNA from each tissue sample. Universal primers (fD1: 5-AGAGTTGATCCTGGCTCAG-3 and Rp2: 5-

ACGGCTACCTTGTTACGACTT-3) were used to amplify the 16S rRNA gene (V3-V4) in preparation for sequencing. The extracted 16S rRNA samples were sent to Genewiz (South Plainfield, New Jersey) in order to be sequenced.

Data analyses

Once the raw sequences data was received, we determined the relative abundance of phylum and family level for each manatee as well as for the collective greater and lesser curvature samples. This allowed for comparison of the stomach tissue microbiota at OTU level between the two different stomach sample locations. The species diversity of the stomach microbiome was evaluated for each individual manatee, lesser curvature, greater curvature, and combine lesser curvature and greater curvature through Shannon's index, evenness, and Simpson's index.

Results

There were 13833 species identified from each sample of the greater curvature in a total of 76 operational taxonomical units (OTUs) in 5 phyla. There were 35272 species identified from each sample of the lesser curvature in a total of 141 OTUs in 10 phyla. Combined, there were 147315 species identified within 217 OTUs in 10 phyla. The highest relative abundance was Firmicutes in lesser curvature (68.802), greater curvature (68.852), and in the combined lesser and greater curvature (68.816) (Table 2). The second highest relative abundance was Bacteroidetes in the greater curvature (22.266) and combined lesser and greater curvature (14.398) but was third highest in lesser curvature (11.313) (Table 2). The second highest relative abundance in lesser curvature was Proteobacteria (14.744) (Table 2). Some of the species identified were not able to be classified within a phyla, but were identified as within the Kingdom Bacteria. There was also species that could not be identified at Kingdom, thus are

labeled as Unclassified (Table 1). Over half, 6, of the phyla identified were not very prevalent within the microbiome (<1%) (Table 2).

Table 2: Relative abundance of bacterial phyla detected for the combined total, combined lesser curvature, and combined greater curvature from the stomach tissue samples from three Florida manatees.

Phylum	Total	Lesser	Greater
Firmicutes	68.816	68.802	68.852
Bacteroidetes	14.398	11.313	22.266
Proteobacteria	11.782	14.744	4.229
Fusobacteria	3.435	3.159	4.137
Actinobacteria	0.697	0.767	0.516
Unknown Bacteria ¹	0.477	0.664	0.00
Acidobacteria	0.136	0.189	0.00
Cyanobacteria	0.080	0.112	0.00
Chloroflexi	0.039	0.055	0.00
Unclassified ²	0.033	0.045	0.00

1. Species could not be identified more specifically than kingdom level of classification
2. Species could not be classified of which kingdom it belongs within

Majority of the OTUs classified could not be identified past the Family level, thus we viewed which Families were most abundant. The top 10 most abundant Families were not consistent between sampling sites, thus greater curvature, lesser curvature, and combined lesser curvature and greater curvature are presented in three separate tables (Table 3-5). Families that were included in all three included, *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Fusobacteriaceae*, *Prevotellaceae*, *Fusobacteriaceae*, and Order Clostridiales (which could not be identified past order) (Tables 3-5). Four of the ten most abundant families in the lesser curvature and the combined belong to the phylum Firmicutes. Five of the ten most abundant families in the greater curvature are Firmicutes. The greater curvature and combined had 3 of the 10 families that are Bacteroidetes while lesser curvature only had two of the 10. The lesser curvature had 4 out of 10 families belong to Proteobacteria, which was the second highest

Table 3: Top 10 bacterial families and respective relative abundance detected from the combined greater curvature and lesser curvature of stomach tissue samples from three Florida manatee.

Clostridiaceae	26.938
Lachnospiraceae	17.012
Ruminococcaceae	16.972
Prevotellaceae	7.319
Fusobacteriaceae	3.410
Sphingomonadaceae	2.929
Vibrionaceae	2.282
Bacteroidaceae	2.254
Order Clostridiales ¹	2.202
S24-7	2.150

1. Species could not be identified more specifically than order level of classification

Table 4: Top 10 bacterial families and respective relative abundance detected from the combined lesser curvature of stomach tissue samples from three Florida manatee.

Clostridiaceae	29.437
Ruminococcaceae	18.653
Lachnospiraceae	15.264
Prevotellaceae	6.023
Sphingomonadaceae	4.078
Vibrionaceae	3.176
Fusobacteriaceae	3.124
Order Clostridiales ¹	3.066
Desulfovibrionaceae	1.757
Bradyrhizobiaceae	1.710

1. Species could not be identified more specifically than order level of classification

Table 5: Top 10 bacterial families and respective relative abundance detected from the combined greater curvature of stomach tissue samples from three Florida manatee.

Lachnospiraceae	21.468
Clostridiaceae	20.567
Ruminococcaceae	12.685
Prevotellaceae	10.624
Order Clostridiales ¹	5.154
Bacteroidaceae	4.877
Veillonellaceae	4.742
Fusobacteriaceae	4.137
S24-7	3.648
Desulfovibrionaceae	2.289

1. Species could not be identified more specifically than order level of classification

relative abundant phyla (Table 2). The top three highest relative abundance families for the combined, lesser curvature, and greater curvature were Clostridiaceae, Lachnospiraceae, and Ruminococcaceae (Tables 3-5).

The Shannon index and evenness shows that diversity is less in manatee 3, with lower Shannon index and lower evenness compared to manatee 1 and manatee 2 (Table 6). Simpson's index supports the same conclusion, in which manatee 1 is slightly less diverse than manatee 1 and manatee 2 (Table 6). Shannon's Index and evenness shows no great difference between lesser curvature, greater curvature, and combined curvature diversity (Table 6). Simpson's Index also shows that there is not a high difference in diversity between lesser curvature, greater curvature, and combined curvature diversity (Table 6).

Table 6: Alpha diversity indices calculated as Shannon index, Evenness, and Simpson's Index. Data displayed are combined total, combined lesser curvature, combined greater curvature, and each individual manatee samples.

	Shannon's Index	Evenness	Simpson's Index
Total	4.3367	0.8061	0.9771
Lesser Curvature	3.8399	0.7759	0.9628
Greater Curvature	3.4928	0.8065	0.9541
Manatee 1	3.7331	0.7912	0.9571
Manatee 2	3.1583	0.7109	0.9352
Manatee 3	2.9754	0.6534	0.9043

Discussion

This study characterized the stomach tissue microbiome of three recently deceased manatees. The highest abundance for phyla in lesser curvature (68.8%), greater curvature (68.9%), and combined lesser and greater curvature (68.8%) was Firmicutes. The second highest phylum abundance in the greater curvature (22.3%) and combined lesser and greater curvature (14.4%) was Bacteroidetes. A lower gastrointestinal tract study of captive Antillean manatee

revealed the same most abundant phyla of Firmicutes (84.1%) and Bacteroidetes (8.6%) (Suzuki et al., 2019). Similarly, in wild Florida manatee, Firmicutes (77.3%) and Bacteroidetes (19.5%) were the most abundant phyla (Merson et al., 2014). In wild dugongs, the most abundant phyla is Firmicutes (75.6%) followed by Bacteroidetes (19.9%) (Eigeland et al., 2012). Firmicutes (83.1%) and Bacteroidetes (15.0%) were found to be the highest two abundant phyla in captive dugongs (Tsukinowa et al., 2008). While the abundance was lower in the stomach tissue samples for Firmicutes than previously studies, the relative abundance of Bacteroidetes is within the range of recorded data. Despite the slight difference in abundance, our data is consistent with previous lower gastrointestinal tract studies of sirenian conducted by Suzuki et al. (2019), Merson et al. (2014), Eigeland et al. (2012), and Tsukinowa et al. (2008).

In addition to our data being consistent with existing data on sirenians, our data is also similar to the findings of the upper gastrointestinal tract studies of recently deceased southern right whale calves, pygmy sperm whales, and dwarf sperm whales. These studies by Maron et al. (2019) and Erwin et al. (2017) have shown to have Firmicutes and Bacteroidetes as the two most abundant phyla. These whales are the closest marine mammal to the Florida manatee that have had their upper gastrointestinal tract studied. Despite their different diets, the composition of their microbiome is similar at this taxonomic level.

Other herbivorous mammals have had their upper gastrointestinal tract investigated. In a study of horses, Firmicutes and Bacteroidetes were also found to be in the highest abundance (Dougal et al., 2013). In a study of feral horses, three of the four most abundant bacterial species are Firmicutes, while the fourth species belongs to Proteobacteria (St-Pierre et al., 2012). The second most abundant phylum in the lesser curvature of the stomach tissue samples is also Proteobacteria (14.7%).

Two elephants, a three-week-old and a six-year-old, showed slight variation in their top abundant phyla of their hindgut. Within the three-week-old, Bacteroidetes (>50%) was predominantly found, followed by Proteobacteria (32%) and then Firmicutes (11%) (Ilmberger et al., 2014). In comparison, the six-year-old consisted mainly of Bacteroidetes (47%) and Firmicutes (36%) (Ilmberger et al., 2014). This is an important comparison, since elephants are closest living relatives to sirenians (Domning, 2012). The three most abundant phyla in elephants and the stomach tissue of Florida manatee are equivalent.

There were slight differences among the three manatees in this study when viewing Shannon's index, evenness, and Simpson's index. Manatee 3 showed slightly less diversity than manatee 1 and manatee 2. When comparing lesser curvature and greater curvature, there were no large differences in these indices and evenness. Previous studies of captive Antillean manatee lower gastrointestinal tract have shown similar diversity with Shannon's indices between 4.19 and 4.69 and Simpson's indices between 0.969 and 0.981 (Suzuki et al., 2019). Lower gastrointestinal tract of wild Florida manatees has shown to have a higher Shannon's index, with indices between 4.85 and 5.87 (Merson et al., 2014). However, Simpson's indices were found to be within 0.91 and 0.96, which is similar to this studies Simpson's indices (Merson et al., 2014).

Conclusion

To the best of our knowledge, this study was a first of its kind to evaluate the microbiome within the upper gastrointestinal tract in any sirenians. We saw that there was not a significant difference in diversity between the lesser curvature and greater curvature in the stomach of Florida manatees. Our data also showed that there are similarities between the upper gastrointestinal tract of Florida manatees and other marine mammals and land herbivores.

Further investigation could provide a better understanding of the health of Florida manatees and aid in the protection of these endangered marine mammals.

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References

- Domning, D.P. (2012). Sirenia (Dugongs and Manatees). In eLS, (Ed.)
doi:10.1002/9780470015902.a0001576.pub3
- Dougal, K., de la Fuente, G., Harris, P., Girdwood, S., Pinloche, E. and Newbold, J., (2013).
Identification of a Core Bacterial Community within the Large Intestine of the Horse.
PLoS ONE, doi:10.1371/journal.pone.0077660
- Eigeland, K., Lanyon, J., Trott, D., Ouwerkerk, D., Blandshard, W., Milinovich, G., Gulino, L.,
Martinez, E., Merson, S. and Klieve, A., (2012). Bacterial Community Structure in the
Hindgut of Wild and Captive Dugongs (*Dugong dugong*), *Aquatic Mammals*, 38:402-411
- Erwin, P., Rhodes, R., Kiser, K., Keenan-Bateman, T., McLellan, W. and Pabst, D., (2017). High
Diversity and Unique Composition of Gut Microbiomes in Pygmy (*Kogia breviceps*) and
Dwarf (*k. sima*) Sperm Whales. *Scientific Reports*, doi:10.1038/s41598-017-07425-z
- Ilmberger, N., Gullert, S., Dannenberg, J., Rabausch, U., Torres, J., Wemheuer, B., Alawi, M.,
Poehlein, A., Chow, J., Turaev, D., Rattei, T., Schmeisser, C., Salomon, J., Olsen, P.,
Daniel, R., Grundhoff, A., Borchert, M. and Streit, W., (2014). A Comparative
Metagenome Survey of the Fecal Microbiota of a Beast- and a Plant-Fed Asian Elephant
Reveals an unexpectedly High Diversity of Glycoside Hydrolase Family Enzymes. *PLoS
ONE*, 9(9).
- Laist, D.W. and Reynolds, J.E., (2005). Florida Manatees, Warm-Water Refuges,
and an Uncertain Future. *Coastal Management*, 33:279-295.
doi:10.1080/08920750590952018
- Laist, D.W. and Reynolds, J.E., (2006). Influence of Power Plants and Other
Warm-water refuges on Florida Manatee. *Marine Mammal Science*, 21(4).
doi:10.1111/j.1748-7692.2005.tb01263.x
- Lefebvre L, Marmontel M, Reid P, Rathbun G and Domning D., (2001) Status and biogeography
of the West Indian manatee. *C.A. Woods and F.E. Sergil, eds. Biogeography of the West
Indies, patterns and perspectives*. Second edition CRW Press, Boca Raton, Pages:425-
474.
- Maron, C., Kohl, K., Chirife, A., Martino, M., Fons, M., Navarro, M., Beingesser, J., McAloose,
D., Uzal, F., Dearing, M., Rowntree, V. and Uhart, M., (2019). Symbiotic microbes and
potential pathogens in the intestine of dead southern right whales (*Eubalaena australis*)
calves. *Anaerobe*, doi:10.1016/j.anaerobe.2019.01.003
- Merson, S., Ouwerkerk, D., Gulino, L., Klieve, A., Bonde, R., Burgess, E. and Lanyon, J.
(2014). Variation in the hindgut microbial communities of the Florida manatee,
Trichechus manatus latirostris over winter in Crystal River, Florida. *FEMS Microbiology
Ecology*, 8:601-605
- Parsons, E.C., Bauer, A., McCafferty, D., Simmonds, M.P., & Wright, A.J. (2013),
Sirenia. In *An Introduction to Marine Mammal Biology and Conservation*, Pages:101-
113. Burlington: Jones & Bartlett Learning.
- St-Pierre, B., de la Fuente, G., O'Neill, S., Wright, A. and Al Jassim, R., (2013). Analysis of
stomach bacterial communities in Australian feral horses. *Molecular Biology Reports*,
40:369-376

Suzuki, A., Ueda, K., Segawa, T. and Suzuki, M., (2019). Fecal microbiota of captive Antillean manatee *Trichechus manatus manatus*. *FEMS Microbiology Letters*, 366.

Tsukinowa, E., Karita, S., Asano, S., Wakai, Y., Oka, Y., Furuta, M. and Goto, M., (2008). Fecal microbiota of a dugong (*Dugong dugong*) in captivity at Toba Aquarium. *General Applied Microbiology*, 54:25-38.