IDENTIFYING THE RELATIONSHIP BETWEEN THE GUT MICROBIOME AND INFLAMMATORY BOWEL DISEASE IN CAPTIVE ADULT RED WOLVES

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Identifying the Relationship between the Gut Microbiome and Inflammatory Bowel Disease in the Captive Red Wolf (*Canis rufus)*

A thesis submitted partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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Captive management of many wildlife species has proven to be challenging, with individuals displaying health disorders that are not generally described in the wild population. Retrospective studies have identified gastrointestinal (GI) diseases, in particular inflammatory bowel disease (IBD), as the second leading cause of captive adult red wolf (*Canis rufus)* mortality. Recent molecular studies show that imbalanced gut microbial composition is tightly linked to IBD in the domestic dog. The goal of the present study was to answer two main questions: (1) How do red wolf gut microbiomes differ among fecal consistency scores? and (2) How do red wolf gut microbiomes differ among diet types? Fresh fecal samples were collected from 53 captive wolves housed in eight facilities and from two wild wolves living in Alligator River National Wildlife Refuge. Each individual sample was given a fecal consistency score (FCS) as a

proxy for GI health. Diet type was of interest due to the influence it can have on the gut microbiome. Gut microbiome composition from each sample was characterized using a targeted amplicon sequencing approach with the 16S rRNA gene. A higher relative abundance of the bacterial phylum Bacteroidetes was observed in samples obtained from captive wolves compared to the samples from wild wolves. Additionally, an increase in relative abundance of Bacteroidetes and a decreased relative abundance in Firmicutes was seen in red wolves with an FCS of 0 compared to wolves with an FCS of 2. In summary, there are differences in gut microbiome composition between a FCS of 0 and a FCS of 2 and among wild, whole meat, mixed and kibble diet types. Findings from this study increase the understanding of the interplay between diet and GI health in the red wolf, a critical piece of information needed to maintain healthy wolves in this captivity sustained species.

CHAPTER 1

THE RED WOLF

First identified by Audubon and Bachman in 1851, the red wolf (*Canis rufus)* is a native canid that historically thrived in the southeastern portion of the United States[8]. Red wolves fall under the order Carnivora and family Canidae which also includes animals like foxes (*Vulpes spp*.), jackals (*Canis spp*.), grey wolf (*Canis lupus),* domestic dogs (*Canis lupus familiaris*) and African wild dogs (*Lycaon pictus)*[42].

Unfortunately, there is not much knowledge of red wolf native habitat because humans decimated the population by the time the species peaked scientific interest[47]. The widespread historic distribution of the species leads scientists to infer that red wolves were successful because of their ability to use more than one type of habitat simultaneously[47]. Anthropogenic forces, such as habitat loss and persecution, applied tremendous pressure that by the 1930's red wolves disappeared from the area east of the Mississippi river[8]. As the number of wild red wolves continued to decline, United States Fish and Wildlife Service (USFWS) collaborated with Point Defiance Zoo and Aquarium to begin captive breeding efforts of the red wolf in 1969[60]. By 1970, there was only one small population of approximately 100 wolves that lingered in coastal portions of

southeastern Texas and Louisiana[60]. This final population was subjected to the anthropogenic threats as well as high parasite infestation rates and hybridization with coyotes (*Canis latrans*) as they began to take over the wolves' range[8]. In 1973, USFWS listed the species as endangered under the Endangered Species Act. In response to the listing, USFWS captured the remaining free-ranging wolves in an effort to preserve the species genetics[61].

From 1973 to 1980, USFWS captured approximately 400 canids and morphologically analyzed to determine if individuals were true red wolves, a red wolf-coyote hybrid or an alternate species. Out of the 400 animals captured, UWFWS biologists determined 43 canids to be pure red wolf and out of those 43, only 14 of them went on to be founders of the captive breeding population[28]. USFWS removed the pure individuals from their native habitat and declared the red wolf extinct in the wild in 1980[28].

The goal of the captive program is to provide a stock of individuals to breed, increasing the population size of red wolves for eventually reintroduction back into parts of its former range. The Red Wolf Recovery Team planned a test reintroduction in western Kentucky and Tennessee at an area called Land Between the Lakes in the early 1980's. A reintroduction plan was drafted for the area but large amounts of backlash from local livestock farms and associations forced the cancellation of the plan[60]. In 1987, USFWS released eight captive born red wolves (four breeding pairs) in Alligator River National Wildlife Refuge (ARNWR)[28] in North Carolina. Initially, the mortality rates for all wolves released

was high due to car strikes, disease or affinity for developed areas[28]. The wolves were kept in an acclimation pen with limited human contact prior to release for generally 19 months[46]. However, the acclimation protocol did not lessen their tolerance of humans from their prior captive environment[46]. Therefore, the reintroduction efforts were slow to produce an established population. To counterbalance the slow progress, USFWS released more than 60 wolves from 1987 until 1994[28]. The recovery area for the red wolf started at 480 km^2 in size then increased to 6,800 km^2 as the population increased[28]. By the mid 1990's, the wolves began to form packs and territories and breed successfully, making this the first successful large carnivore reintroduction[28].

With accomplishments in ARNWR, USFWS did a second reintroduction and placed 37 wolves in the Great Smoky Mountain National Park (GSMNP)[28]. Unfortunately, the reintroduction in this area was halted due to high mortality of adults and pups from starvation, disease, straying outside the park and pressure from bears and coyotes[28]. The remaining wolves in GSMNP were recaptured and relocated to ARNWR[28]. By 2002, the ARNWR population demographic transitioned from captive born to all wild born individuals[60].

The reintroduced population reached its peak of 140 wolves in 2001 but going forward numbers continuously decreased because of various threats including gunshot, vehicle collision and disease[28]. Anthropogenic forces accounted for 40.6% of red wolf mortality in ARNWR, with gunshot being the most common cause of death[26]. Many of the gunshot mortalities stemmed from

misidentification while legally hunting coyotes[40]. As of 2019, there are around 25 red wolves that still inhabit ARNWR[66].

NATURAL HISTORY

The natural diet of red wolves consists of mostly white tail deer (*Odocoileus virginianus*), rabbit (*Sylvilagus spp.*), raccoons (*Procyon lotor*), small mammals like nutria (*Myocastor coypus*) and various rodents[60]. On a daily basis, an adult consumes two to five pounds of food and will roam up to 20 miles in search of food[60]. The home range of red wolves varies from 25 km^2 to 190 km^2, with the core range varying from 1.9 km^2 to 20.5 km^2 [29]. The range for an individual remains consistent during all seasons[2]. Red wolves breed with the same mate for life, unless the breeding pair is disrupted by mate death[30]. Like the grey wolf, the red wolf lives in multi-generational packs consisting of the adult breeding pair and their offspring from various years[60]. Juvenile wolves will disperse from their family pack around two years of age[60].

RED WOLVES IN CAPTIVITY

Currently, there are 257 red wolves living in 42 facilities as part of the Association of Zoos and Aquarium's Species Survival Plan (SSP). The SSP is a cooperative action where participating institutes oversee management of select species with the goal of maintaining sustainable long term populations[54].

Gastrointestinal disease is the most common cause of death for ex situ adult red wolves[1] directly killing 21% of captive adult red wolves from 1992 through 2012, with IBD specifically causing mortality in 25% of wolves[1,53]. Additionally, non-lethal GI lesions were found in 25% of wolves that died from 1992-2012, with IBD being found in 59%[1,53].

Environmental factors, like captive diet, can be one piece of the puzzle contributing to the cause(s) of IBD in red wolves. In captivity, red wolves are fed a high energy, nutritionally balanced, meat-based dry dog food approved for domestic dogs[4]. According to the AZA guidelines, the red wolf diet should consist of 90-95% base diet, or dry dog food, and can include 5-10% of supplemental feed like prepared meat, bones or carcasses[4]. Red wolves are fed six out of the seven days of the week except when young are present or periods of sustained cold temperatures (~4.4°C); however, this can vary from facility to facility[4]. Additionally, the wolves are fed once a day with the time of day being determined by each institution, yet this also can vary from facility to facility[4]. Research into the root cause of gastrointestinal inflammation can provide knowledge that will benefit the health, management and survival of captive red wolves.

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease is a phrase used to describe multiple diseases of which origins are unknown but are becoming increasingly prevalent around the world in humans, canids and felids[41,57]. These diseases are

characterized by inflammation in the small or large intestinal mucosa and are frequently the cause of chronic vomiting and diarrhea in canines[60]. This term includes diseases like ulcerative colitis, which almost exclusively affects the colon and mucosa, and Crohn's disease, which can occur anywhere along the GI tract involving transmural inflammation[44]. Histological evidence is needed to confirm diagnosis to distinguish IBD from other GI disorders including viral infection, parasites, intestinal tumor or mechanical obstruction[12,19]. Although the etiology is unknown, there is strong evidence that gut microbiota plays an integral part in the development of IBD, along with environmental factors and genetic predisposition of the host[27,58,70].

In examining IBD in red wolves, one can utilize well-studied species like the mouse and humans, but the best evidence for the etiology of the disease in wild canids is to use domestic dogs as a model species. For instance, the three most common types of chronic intestinal inflammation have been described in domestic dogs[19]. Lymphocytic, plasmacytic and eosinophilic enteritis are common intestinal inflammation characterized by invasion of lymphocytes, plasma cells and white blood cells respectively, into the intestinal mucosa[19]. In dogs, lymphocytic and plasmacytic enteritis are the most common types with lesions occurring mostly in the small intestine[19]. Additionally, many forms of IBD are exclusive to particular dog breeds, such as histiocytic ulcerative colitis in Boxers[37,59], immunproliferative enteropathy of Basenjis, protein-losing enteropathy and associated protein-losing nephropathy in Soft Coated Wheaten

Terriers. Spontaneous lymphoplasmacytic, eosinophilic gastritis and enteritis have all been seen in the captive red wolf population[53].

Henson et al[27] uncovered that red wolves have a genetic predisposition to IBD. Domestic dogs carry three single nucleotide polymorphisms (SNP) in toll-like receptor (TLR) 5 that are associated with IBD; G727A, C805T and C2549T[35]. Because the protective thymine allele in both C805T and C2549T that is consistent across all domestic dog breeds[27] is not possessed by captive red wolves, it has been suggested that this species has a genetic predisposition to IBD. Additionally, TLR5 is a critical part in the gut microbiota-immune system relationship because of its ability to identify bacteria flagellin as commensal or pathogenic[27]. The stimulation of TLR can result in the excretion of proinflammatory mediators and transcription of inflammatory and immunoregulatory genes[36]. However, a sustained stimulation of TLR can prompt tissue injury and epithelial cell death[36]. Domestic dogs and humans with IBD have an increased mucosal expression of TLR2, TLR4 and TLR5 genes[35].

GUT MICROBES

The GI tract is filled with large populations of microbes that protect against pathogens, ferment non-digestible dietary particles and aid in proper immune function[44]. Microbes protect against pathogen establishment by inhibiting the entry into epithelial cells, where they would compete for nutrients and space, and generate antimicrobial material[44]. Microbes can also metabolize the epithelial

cells that are shed, GI tract mucosa and non-digestible material that comes through the small intestine[44]. As these nutrients are fermented, they produce short-chain fatty acids (SCFAs) that in turn are an energy source for epithelial cell growth and bacterial metabolism[44].

The gut microbiota acquires a majority of their nutrients via carbohydrates in the host's diets. Fermentation of these carbohydrates is done by colon bacteria in the genus *Bacteroides*, *Roseburia*, *Bifidobacterium*, *Fecalibacterium* and *Enterobacteria* which produces SCFA like butyrate, propionate and acetate[32]. Individuals within the genus Bacteroides are the bacteria that perform most of the carbohydrate digestion using enzymes like glycosyl transferase, glycoside hydrolases and polysaccharide lyases[32]. Another key function of gut microbiota is the synthesis of vitamin K and B[32].

SCFAs moderate innate and adaptive immune cell production, movement and function[20]. Butyrate, a SCFA, possesses anti-inflammatory effects by halting the gathering and inflammation boosting activities of neutrophils, macrophages, dendritic cells and effector T cells while increasing the abundance and activity of regulatory T cells[20]. Dogs with IBD have gut dysbiosis characterized by a decrease in SCFA-generating bacteria, typically the phyla Firmicutes and Bacteroidetes[20].

A healthy gut has the capacity to mitigate its immune response to antigens from food and commensal bacteria while sustaining the capability to appropriately respond to pathogens[23]. The GI tract of a healthy domestic dog is

home to bacteria phyla Firmicutes[31], Proteobacteria[31], Bacteroidetes[31], Spriochaetes[22,69], Fusobacteria[31] and Actinobacteria[22,69]. Mostly the genus *Clostridia* bacteria inhabits the duodenum and jejunum while Fusobacteria and Bacteroides bacteria are dominant in the ileum and colon[22]. Genus *Lactobacilli* is commonly found in all parts of the canine intestine, with *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus* and *Lactobacillus salivarius* being the most common species in a healthy canine[22].

Studies of dogs with IBD demonstrate a variety of changes in the gut microbiome. Dogs with IBD may have an increase in bacteria from the phyla Firmicutes[22,59,69], Proteobacteria[39] and Actinobacteria[22,59,69] with a decrease in Bacteriodetes[10,39,44], Fusobacteria[22,59,69] *Lactobacillus*[22,39,57,70] and *Clostridiales*[22,39,57,70] in the GI tract. In contrast, a couple studies document a decrease in Firmicutes[10,39,57] in the GI tract in association with IBD in dogs. I expect that red wolves will show similar changes in their microbiome as a result of IBD, but it has never been systematically tested.

The alteration to native commensal bacterial community within the gut, or gut dysbiosis, can be caused by various influences like diet, infection, antibiotics or genetics of the host[45]. There are three types of dysbiosis; the decrease of beneficial microbes, growth of possibly harmful microbes or the decrease of overall microbial diversity[45]. The three types are not mutually exclusive[45]. For the present study, the focus will be on how gut dysbiosis is influenced by specific environmental factors like diet type.

CHAPTER 2

PROJECT JUSTIFICATION

The red wolf (*Canis rufus)* is a critically endangered North American canid that historically inhabited the south eastern United States[28]. The species was nearly exterminated by predator control programs and continues to face threats of hybridization with coyotes and human caused mortality[61]. Today, red wolves exist in *ex situ* populations and one small reintroduced population in Alligator River National Wildlife Refuge (ARNWR) in North Carolina[47,61,62]. The *ex situ* population serves to maintain the genetic diversity of the red wolf and is critical for the continued existence of the species[26]. Unfortunately, *ex situ* populations are threatened by health issues or genetic diseases that are not found in the wild population[1].

Gastrointestinal (GI) disease is the second most common cause of mortality in the adult captive red wolf from 1992 through 2012, killing 32 out of 151 (21%) wolves[1,53]. Specifically, inflammatory bowel disease (IBD), a type of GI disease, is the cause of mortality in 8 out of the 32 wolves (25%) that died from GI diseases[1,53]. Furthermore, an additional 37 out of 151 (25%) wolves that died (1992 through 2012) had non-lethal GI lesions, and 68% (22 out of those

37) of those wolves had IBD[1,53]. Since 2018, there were four deaths within the SSP population due to GI issues[68]; for instance, one male died from gastric perforations which can be caused by IBD[68]. The use of endoscopy and/or histology has shown that it is common for wolves in the SSP population to have duodenum lesions[68].

It has recently been discovered that the red wolf may have a genetic predisposition to IBD[27]. Captive red wolves do not possess the protective thymine allele in two single nucleotide polymorphisms (SNP) in the toll-like receptor (TLR) 5 that are associated with IBD in the domestic dog; the absence of which presents a genetic predisposition[27]. TLR5 plays a critical role in the cross-talk between the gut microbiota and the immune system because of its ability to identify bacteria as commensal or pathogenic[27]. The activation of TLR can cause an excretion of pro-inflammatory mediators but, a prolonged activation of TLR can prompt tissue injury and epithelial cell death[27]. The causative agent of red wolf GI disease remains unknown; however, it is probable that complex interactions between GI microbiota, environmental factors and a concealed genetic predisposition play a role[57]. An imbalance in the community structure of gut microbiota, termed gut dysbiosis, is a common pattern and has been linked to GI diseases in humans and canines[45,70,71]. Further, gut microbiota is linked to diet type in humans[32] and canids[58].

The GI tract is filled with large populations of microbes that protect against pathogens, ferment non-digestible dietary particles and aid in development of the

immune system[44], among many other functions. In recent years, studies have demonstrated the relationship between bacteria in the gut and the improvement of GI health and/or GI diseases has been established[38]. Changes in the gut microbial community that promote disease, termed gut dysbiosis, are linked to irritable bowel syndrome, acute diarrhea and IBD in humans and canids[5,58]. Gut dysbiosis can be provoked by various factors like diet, infection, antibiotics or genetics of the host[45]. For example, domestic dogs with acute hemorrhagic diarrhea possess a lower relative abundance of the phylum Actinobacteria and various members of the phylum Firmicutes, specifically the family *Ruminococcaceae*[58]. In the GI track of dogs with IBD there may be a relative increase of bacteria from the phyla Proteobacteria[39] and Actinobacteria[22,59,69] with a decrease in relative abundance of Bacteriodetes[39,44] and Fusobacteria[22,59,69] , and either an increase[22,59,69] or a relative decrease in the phylum Firmicutes[39,57]. The phylum Firmicutes plays a role in the production of short chain fatty acids (SCFA) which can benefit GI health, protect against intestinal inflammation and advantageously regulate permeability of the intestinal membrane[58].

To date, there is no information about the link between gut microbiota and GI health in the red wolf. The overall goal of this study was to characterize the gut microbiome of adult captive red wolves and its relationship to GI health. My main questions were, how do gut microbiomes in the red wolves differ among 1) fecal consistency scores and 2) diet types. I hypothesized that the gut

microbiome composition differed between animals with varying fecal consistency scores, considered a proxy of GI health[33]. I also hypothesized that gut microbiome composition differed based on diet type. I expected that red wolves would show similar changes in the gut microbiome due to IBD, but it has never been systematically tested. For the present study, I focused on how gut dysbiosis was influenced by specific environmental factors like diet type. The present study was the first to characterize the gut microbiome structure of the red wolf and assess the association of fecal consistency scores and diet type on the gut microbiome composition. Histological evidence is needed to confirm diagnosis of IBD[57]; therefore, GI health status is hard to directly measure because of sample collection invasiveness. While chronic diarrhea/soft stool is not a unique sign of IBD, it gives reason to believe that a GI issue is occurring in that individual. For the present study, FCS was one component of the best proxy available for a non-invasive way to estimate GI health. Gastrointestinal diseases, such as IBD are a serious threat to the ex situ population of red wolves. These remaining red wolves in captivity are critical to the survival of this species as they make up a large portion of the small population left in the world.

MATERIALS AND METHODS

ANIMALS

Fecal samples were opportunistically obtained from 50 red wolves in total, 48 *in situ* housed in eight facilities and 2 *ex situ (*Table 1)*. Ex situ* wolves had one

of the following diets: 1) kibble-based diet comprised of a high energy, meat-based dry dog food approved for domestic dogs (hereby referred to as kibble). Generally, the brands fed were Hill's Science Diet Canine Active, Red Flannel, Taste of the Wild High Prairie Grain Free, Taste of the Wild Pacific Stream Grain Free, Purina Complete Adult Dog Chow and Mazuri Canine Chow, 2) mix of kibble and commercial meat, typically Nebraska brand Classic Carnivore Diets – canine or feline log comprised largely of horse meat ('mixed') or, 3) a whole meat diet which consisted of donated or roadkill white tail deer, elk, wild turkey, beaver, rats, guinea pig or chicken ('whole meat'). In this study, I did not conduct a diet analysis on the wild samples. However, red wolves primarily consume white tail deer, rabbit, raccoons, small mammals and various rodents[60], they were labelled as the 'wild' diet category. Individuals categorized as the kibble diet category were fed dry kibble for 5 out of 7 days of the week. The mixed diet category was defined as a mix of kibble and commercial meat for 5 out of 7 days of the week. The whole meat and wild categories were defined as consuming carcass or whole meat for 5 out of the 7 days.

FECAL SAMPLE COLLECTION AND SCORING

A total of 63 fecal samples were collected from captive wolves (n=48) opportunistically by keepers during routine physical examinations or within one hour of visual observation of defecation. The project was reviewed by the Smithsonian National Zoological Park's IACUC committee and George Mason University's IACUC committee. This study was found to be exempt due to its

non-invasive nature. Samples were labeled with the animal's studbook number, date and facility and then kept at -20°C until overnight shipment to the Smithsonian Conservation Biology Institute (SCBI). Once samples arrived at SCBI they were stored at -80°C until extraction. Two fecal samples were opportunistically collected from *in situ* wolves during 2018 trapping efforts of the remaining wild population conducted by US Fish and Wildlife Service in ARNWR in Manteo, North Carolina.

At SCBI, I scored the fecal consistency of each fecal sample once thawed prior to extraction based on previously described criteria: 0=normal/slightly soft feces, 1=soft feces with or without blood and/or mucus, 2=very soft feces and 3=watery diarrhea[33].

Table 1. List of each participating wolf, the SSP facility and its location, the age, sex and number of samples collected from each wolf and their diet type. Total number of samples is 67.

Studbook number	Facility	Location	Age	Sex	Number of samples	Diet type
1582	Fossil Rim Wildlife Center	Glen Rose, TX	10	F	1	Kibble
1583	Fossil Rim Wildlife Center	Glen Rose, TX	10	F	1	Kibble
1581	Fossil Rim Wildlife Center	Glen Rose, TX	11	M	1	Kibble
2112	Fossil Rim Wildlife Center	Glen Rose, TX	3	F	2	Kibble
2118	New York Wolf Conservation Center	Salem, NY	2	M	1	Whole meat
1858	North Carolina Museum of Life and Science	Durham, NC	6	F	1	Mixed
2210	North Carolina Museum of Life and Science	Durham, NC	6 months	F	1	Mixed
1803	North Carolina Museum of Life and Science	Durham, NC	8	M	1	Mixed
2062	North Carolina Museum of Life and Science	Durham, NC	4	F	1	Mixed
2247	North Carolina Museum of Life and Science	Durham, NC	8 months	M	2	Mixed
2246	North Carolina Museum of Life and Science	Durham, NC	8 months	M	1	Mixed
2079	Northeastern Wisconsin Zoo	Green Bay, WI	4	M	12	Mixed
2081	Northeastern Wisconsin Zoo	Green Bay, WI	4	M	1	Mixed
1931	Point Defiance Zoo and Aquarium	Tacoma, WA	6	F	1	Kibble
1935	Point Defiance Zoo and Aquarium	Tacoma, WA	6	F	1	Whole meat
1585	Point Defiance Zoo and Aquarium	Tacoma, WA	10	F	1	Kibble
2153	Point Defiance Zoo and Aquarium	Tacoma, WA	2	F	1	Kibble

1807	Point Defiance Zoo and Aquarium	Tacoma, WA	7	F	1	Kibble
1491	Point Defiance Zoo and Aquarium	Tacoma, WA	6	M	1	Kibble
1381	Point Defiance Zoo and Aquarium	Tacoma, WA	13	M	1	Kibble
1943	Point Defiance Zoo and Aquarium	Tacoma, WA	5	M	1	Kibble
1942	Point Defiance Zoo and Aquarium	Tacoma, WA	5	M	1	Kibble
1946	Point Defiance Zoo and Aquarium	Tacoma, WA	6	F	1	Whole meat
2003	Point Defiance Zoo and Aquarium	Tacoma, WA	5	F	1	Kibble
1416	Point Defiance Zoo and Aquarium	Tacoma, WA	13	F	1	Kibble
2078	Point Defiance Zoo and Aquarium	Tacoma, WA	3	F	1	Kibble
1363	Point Defiance Zoo and Aquarium	Tacoma, WA	14	F	1	Kibble
2139	Point Defiance Zoo and Aquarium	Tacoma, WA	2	F	1	Kibble
1861	Point Defiance Zoo and Aquarium	Tacoma, WA	6	M	1	Kibble
2007	Point Defiance Zoo and Aquarium	Tacoma, WA	5	F	1	Kibble
1496	Point Defiance Zoo and Aquarium	Tacoma, WA	11	F	1	Kibble
2138	Point Defiance Zoo and Aquarium	Tacoma, WA	2	F	1	Kibble
2076	Point Defiance Zoo and Aquarium	Tacoma, WA	4	M	1	Kibble
2132	Point Defiance Zoo and Aquarium	Tacoma, WA	2	M	1	Kibble

1927	Point Defiance Zoo and Aquarium	Tacoma, WA	6	M	1	Kibble
1415	Point Defiance Zoo and Aquarium	Tacoma, WA	13	F	1	Kibble
1928	Point Defiance Zoo and Aquarium	Tacoma, WA	5	M	1	Kibble
2077	Point Defiance Zoo and Aquarium	Tacoma, WA	4	M	1	Kibble
2006	Point Defiance Zoo and Aquarium	Tacoma, WA	5	M	1	Kibble
2005	Point Defiance Zoo and Aquarium	Tacoma, WA	5	M	1	Kibble
1382	Point Defiance Zoo and Aquarium	Tacoma, WA	13	F	1	Kibble
11600	Sandy Ridge	Manteo, NC	11	F	1	Kibble
11276	Sandy Ridge	Manteo, NC	15	F	1	Mixed
11599	Sandy Ridge	Manteo, NC	11	F	2	Kibble
11473	Sandy Ridge	Manteo, NC	12	F	1	Kibble
1922	Trevor Zoo	Millbrook, NY	5	M	1	Kibble
1479	Trevor Zoo	Millbrook, NY	11	F	1	Kibble
1932	Wolf Haven International	Tenino, WA	5	F	1	Mixed

MOLECULAR METHODS

I extracted bacterial DNA from each sample using the QIAamp PowerFecal DNA Kit (#12530-50, Qiagen, MD) following manufacture's protocol. A sterile 1.5 ml microcentrifuge tube was included for each set of sample extractions as a negative control. I determined DNA concentration and quality on a NanoDrop One (Thermo Scientific, MA).

A two-step polymerase chain reaction (PCR) protocol combined with dual-index paired-end sequencing was used to prepare a library. For the first PCR step (amplicon PCR), I amplified a ~380 base pair region in the V3-V5 region of the 16S rRNA gene using the universal gene primers 515F (GTGCCAGCMGCCGCGGTAA) and 939R (CTTGTGCGGGCCCCCGTCAATTC). Duplicate PCR reactions were done for each sample and included the negative extraction controls and negative PCR controls. The 20 ul amplicon PCR assay consisted of 10 μl of 2x Phusion HotStart II HF Master Mix, 1 μM of the forward primer, 1 μM of the reverse primer and 2 μl of DNA template at 10-15 ng/μl concentration. PCR conditions were: a) activation at 98°C for 30 seconds; b) 25 cycles of denaturation at 98°C for 10 seconds,; c) annealing at 68°C for 20 seconds,; d) extension at 72°C for 30 seconds; and, e) a final extension at 72°C for 5 minutes. I pooled duplicate

amplicon PCR reactions together then performed index PCR, attaching the custom i5 and i7 adaptors during a second PCR step (index PCR) to provide unique identities to each fecal sample. The 50 μl index PCR assay consisted of 25 μl of 2x Phusion Hot Start II HF Master Mix, 5 μl of the i5 primer, 5μl of the i7 primer and 5 μl of cleaned amplicon products. Index PCR conditions were: a) activation at 98°C for 2 minutes; b) followed by 8 cycles of denaturation at 98°C for 20 seconds,; c) annealing at 63°C for 30 seconds,; d) extension at 72°C for 30 seconds,; and, e) a final extension at 72°C for 2 minutes. I used Speed-beads (in a PEG/NaCl buffer)[51] to clean post-PCR products between each PCR reaction and verified PCR products using gel electrophoresis. The concentration of each cleaned index PCR product was measured using a Qubit4 (Invitrogen, MA) and samples were pooled together in equimolar proportion. I ran the pooled library on an E-Gel Power Snap Gel Electrophoresis System (Invitrogen, MA) using a 2% agarose gel cassette and cut out the target band. The library from the gel cut was extracted using a QIAquick Gel Extraction Kit (#28704, Qiagen, MD) and diluted the library to 4 nM. I used real time qPCR, following the KAPA Library Quantification Kit Illumina Platforms protocol, to confirm the concentration of the library (KK4824, Roche Sequencing and Life Sciences, MA) post gel extraction. The pooled library was sequenced on two Illumina MiSeq runs (v3 chemistry: 2x300 bp kit) at the Center for Conservation Genomics, National Zoo.

I imported demultiplexed reads from the Illumina MiSeq into R version 3.5.0[49]. I used the package ‘dada2’ version 1.12[9], to check for chimeras and filter

low-quality sequences (maxEE > 2). I generated amplicon sequence variants (ASVs) and assigned ASVs taxonomy by aligning the sequences against the Ribosomal Database Project (RDP) 16S train set 16/release11.5[63]. I built a phylogenetic tree in the program Quantative Insights Into Microbial Ecology 2 (vQIIME2-2018.4)[7] using the fasttree algorithm[48]. I removed likely contaminant ASVs using the package decontam[13] and the Fisher method with a threshold of 0.01, which removed three ASVs. I then filtered out any negative control samples and singletons ASVs (ASV that occurs as one sequence in one sample). Some individuals (RW2079, RW2112, RW11559 and RW2247) had multiple samples and a random number generator was used to choose which sample was included in the analyses. Only one sample (RW1491) had a FCS of 3, thus it was removed from analyses. The variation in the sequencing depth was approximately 8x (max = 35,296, min = 4,418); therefore, I did not employ normalization correction methods following recommendations by Weiss et al[65]. Any phylum of bacteria with a relative abundance of less than 0.5% was excluded from analyses.

STATISTICAL ANALYSIS

All statistical analyses were conducted in R version 3.5.0 unless otherwise stated and significance was determined as $p < 0.05$. I conducted a Fisher's Exact Test using the function *fisher.test* to determine if there was an association

between FCS and diet type. FCS was considered as a factor for the all analyses unless stated otherwise because each number represented a category of FCS.

I examined if alpha diversity, or within individual diversity, differed between each FCS and diet category using two measures, ASV richness and Faith's Phylogenetic Diversity (PD). ASV richness determined if bacterial taxa richness differed among FCS and diet type while Faith's Phylogenetic Diversity determined how phylogenetically diverse the bacterial community was among FCS and diet type. Within the 'car' package, I conducted a two-way ANOVA with ASV richness or Faith's PD as the response variable and FCS, diet type and facility as the explanatory variables[17]. A Shapiro-Wilk analysis was conducted to test data for normality and a Levene test was conducted to assess homogeneity of variances. I used the function *TukeyHSD* to perform posthoc analyses on each ANOVA that found significance.

To examine beta diversity, or between community diversity, I utilized PERMANOVAs with Jaccard, Bray Curtis or Unifrac distance as the response variable and FCS, diet type and facility as the explanatory variables[43]. This method was appropriate because it is flexible, it works well with zero-inflated data, like DNA sequence data, and it calculates p-values using permutations, negating the assumption of normality[3]. I ensured that dispersion was consistent between samples and was not influencing the PERMANOVA results by conducting a PERMDISP. This analysis helps detect if there are differences in dispersion of the data among the factors of interest, which can lead to false

conclusions of the PERMANOVA. I conducted a mantel test, function *mantel*, using the package 'vegan'[43] to assess for correlations between compositional dissimilarity matrices (Jaccard, Bray-Curtis and Unifrac) and fecal consistency score dissimilarity matrix (Euclidean distance) using 999 permutations. This analysis assessed whether gut microbial community composition became more dissimilar as individuals become more different in FCS.

I used the package 'DAtest' to perform differential ASV abundance testing between FCS and the diet types[52]; this package first ranks statistical methods used to assess differential abundance. Then I used the function *preDA* to filter out low abundance ASVs that were present in less than 12 samples for diet type and less than 10 samples for FCS. I chose these parameters because it removed features where there was not enough information while also retaining the rare features of potential interest[52]. For both FCS and diet type, differential abundance testing was done at ASV and phylum level. This was done to examine if there were any specific ASVs that were differentially abundant among categories while phylum level was assessed because the taxonomic classification was more trustworthy at this level. I then input raw sequence counts of the filtered ASV table or unfiltered phylum level table. I used the *testDA* function to perform the various default transformations of the data based on the statistical method. The three differential abundance tests used had the lowest False Positive Rate for the ASV level[52] for our statistical analyses as the response variable and FCS or diet type as the explanatory variables. I only

reported ASVs, phyla or classes that were significant in two out of the three of the top ranked differential abundance tests. For diet type, the statistical methods used were an ANOVA - Multiplicative zero-correction and additive log-ration normalization (function *DA.aoa)*, Kruskal-Wallis test (function *DA.kru)* and linear regression with multiplicative zero-correction and center log-ration normalization (function *DA.lmc)*. For FCS, the statistical methods used were ANOVA (function *DA.aov)*, Kruskal-Wallis test (function *DA.kru)* and linear regression with multiplicative zero-correction and additive log-ration normalization (function *DA.lma*).

RESULTS

I characterized the gut microbiome of 49 wolves from 8 facilities (Table 2). Samples from 22 wolves had a FCS 0, five wolves had a FCS 1 and 12 wolves had a FCS 2. Thirty-four wolves were categorized under kibble, 10 wolves under the mixed diet, three wolves under whole meat diet and two wolves under the wild diet type. There was no association between FCS and diet type (Fisher's Exact Test, $p = 0.18$).

Table 2. Table illustrates the number of wolves in each fecal consistency score (FCS) category and diet type category. FCS was used as a proxy to measure GI health; 0 which is normal/slightly soft, 1 is soft feces that could include presence of blood or fecal mucosa and 2 is very soft feces. The diet type categories are composed of kibble, mixed kibble and commercial meat, whole meat/carcass and wild type diet.

	Kibble	**Mixed**	**Meat**	**Wild**	**FCS total**
FCS 0	16	4	0	2	22
FCS 1	10	2	3	0	15
FCS 2	8	4	0	0	12
Diet total	34	10	3	2	

I obtained 719,973 high quality sequences from 49 red wolf fecal samples (mean =14,693 sequences, min = 4,418, max = 35,296). A total of 434 ASVs, belonging to five bacteria phyla, *Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria and Fusobacteria*, were identified. I examined relative abundance of each phyla among the three diet types and FCS, respectively (Figure 1).

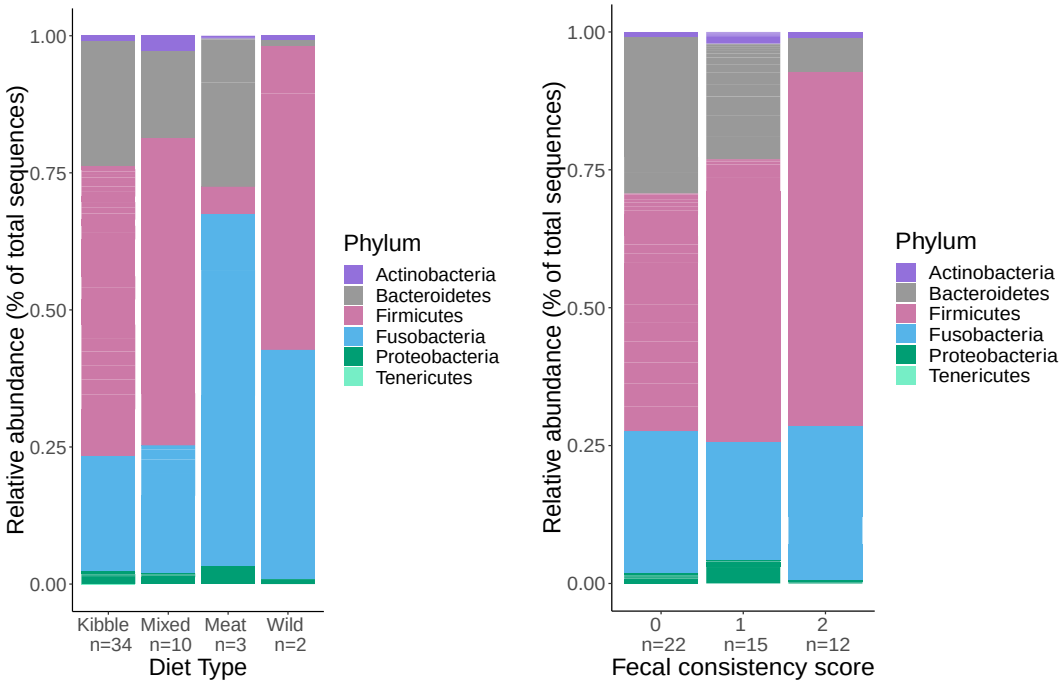


Figure 1. Relative abundance stacked bar plots of top six bacterial phyla found in the gut microbiome of captive and wild red wolves. On the left is the relative abundance of the top six phyla among four diet types; kibble, mixed kibble and commercial meat, whole meat/carcass and wild type diet. Diet type was examined due to the impact it can have on the gut microbiome composition. On the right is the relative abundance of the top six phyla among three fecal consistency scores (FCS); 0 which is normal/slightly soft, 1 is soft feces that could include presence of blood or fecal mucosa and 2 is very soft feces. FCS was used as a proxy to measure GI health.

GUT MICROBIOTA RELATIVE TO DIET TYPE

Diet type influenced changes in the number of bacterial taxa present and the phylogenetic relationship among the gut microbiota. Gut bacterial species richness was significantly different among the four diet types (ANOVA: F_{stat} = 3.475, p = 0.03, df = 3), with individuals consuming a wild diet having a lower

number of bacterial taxa than those consuming a kibble and mixed diet (TukeyHSD: p = 0.02, p = 0.03) (Figure 2). Faith's Phylogenetic Diversity (PD) also differed among diet types (ANOVA: F_{stat} = 6.062 p = 0.002, df = 3), with the wild individuals having lower Faith's PD compared to the kibble diet, mixed diet and whole meat diets (TukeyHSD: p = 0.01, p = 0.002, p = 0.02) (Figure 3).

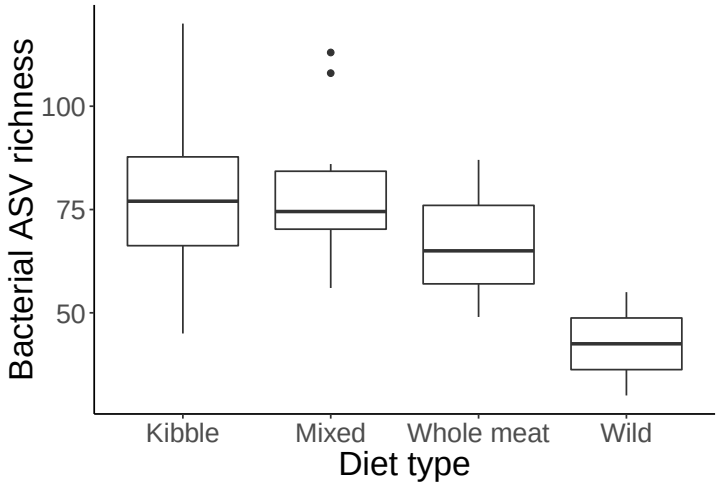


Figure 2. Box plot to visualize gut bacterial ASV, or a proxy for taxonomic richness, in the four different diet types of the captive and wild red wolf. Bacterial ASV richness was used to measure the alpha diversity of the gut microbiome by assessing how many bacterial taxa were present in the gut microbial community. Bacterial ASV richness differed among diet types (ANOVA: Fstat = 3.164, p = 0.034, df = 3); the wild adult red wolf gut microbiome had lower bacterial ASV richness compared to the gut microbiome of captive red wolves fed kibble and mixed diets (Tukey's HSD: p = 0.03)

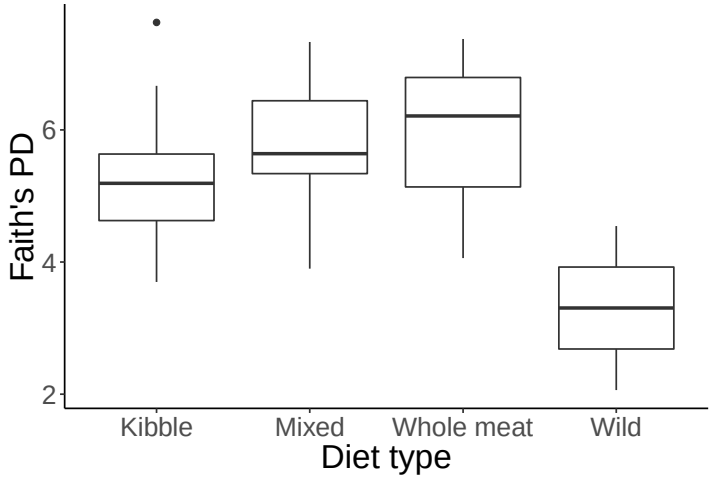


Figure 3. Box plot to visualize gut bacterial Faith's Phylogenetic Diversity (PD) in the four different diet types of the captive and wild red wolf. Faith's PD was used to measure the alpha diversity of the gut microbiome by assessing how phylogenetically diverse the gut bacterial community was. Faith's PD differed among diet types (ANOVA: Fstat = 5.424 p = 0.002, df = 3),) with the wild adult red wolf gut microbiome had lower bacterial ASV richness compared to the gut microbiome of captive red wolves fed kibble, mixed and whole meat diets (Tukey's HSD: p = 0.02, p = 0.003 p = 0.02).

Diet type influenced the bacterial taxa present in the gut microbiome. Different diets impacted the overall bacterial community composition of the red wolf (Figure 4, PERMANOVA: Jaccard Psuedo-F = 2.517, df = 3, R^2 = 13%, p = 0.001; Bray Psuedo-F = 2.2129, df = 3, R^2 = 11.2%, p value = 0.001; unweighted Unifrac Psuedo-F = 3.3508, df = 3, R^2 = 16.1%, p = 0.001), with differences in composition found between wolves that consumed a kibble and whole meat diet (pairwise p <0.05 for Bray-Curtis, Jaccard, Unifrac distance), mixed and whole meat diet (pairwise p <0.05 for Jaccard and Unifrac distance), mixed and kibble diet (pairwise p <0.05 for Jaccard and Unifrac distance) and kibble and wild diet (pairwise p < 0.05 for Jaccard and Unifrac distance). The bacterial communities

of each diet type had dissimilar dispersion (PERMDISP: Jaccard p = 0.01, Bray-Curtis p = 0.01), which may be driving the significant difference in bacterial community among the diet types.

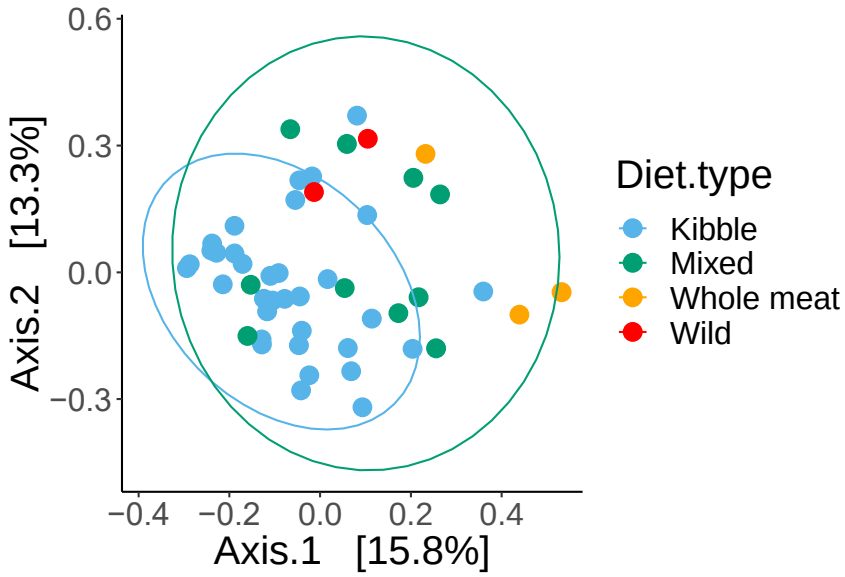


Figure 4 Principal Coordinate Analysis (PCoA) used to visualize the spatial relationship of fecal bacterial community composition among 49 red wolves (Unifrac distance). Although there was some overlap, gut microbial community composition significantly differed among diet types. Specific difference lied between kibble and mixed diet, mixed and whole meat diet, kibble and whole meat diet and wild and kibble diet. 95% confidence ellipses shown for diet types that have greater than 2 samples.

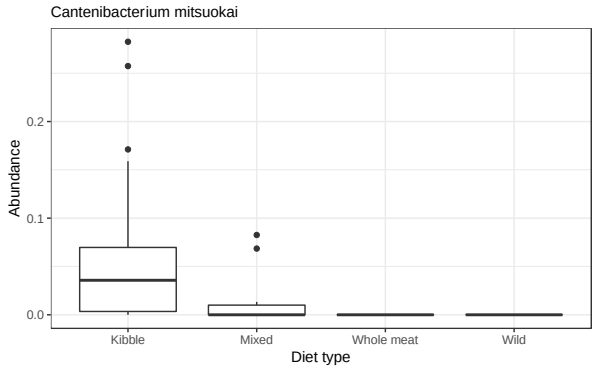
There were no bacterial phyla that were differentially abundant among the four diet types. However, specific bacterial taxa within the phyla could be indicative of certain diet types. For instance, relative abundance of ASV *Catenibacterium mitsuokai,* in the phylum *Firmicutes,* was significantly higher in

wolves fed a kibble diet compared to all other diet types (*DA.aoa* p = 0.003; *DA.lmc* p = 0.01; Figure 5a). A similar pattern was true for another *Firmicutes,* ASV *Holdemanella spp.* (*DA.aoa* p = 0.003; *DA.lmc* p = 0.001; *DA.kru* p = 0.04; Figure 5b). Comparably, relative abundance of the bacterial ASV *Prevotella spp.,* of the *Bacteroidetes* phylum, was significantly higher in kibble fed wolves than mixed diet, whole meat and wild diets (Figure 5c).

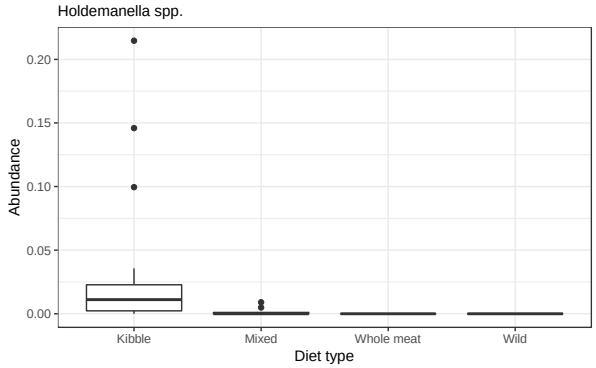
GUT MICROBIOTA RELATIVE TO FECAL CONSISTENCY SCORE

The gut microbiome of red wolves with different FCS were similar in bacterial alpha diversity. There was no significant difference in bacterial ASV richness among the three FCS (ANOVA: $F_{stat} = 1.070$, p = 0.353, df = 2). Similarly, there was no difference in Faith's Phylogenetic Diversity among the three FCS (ANOVA: $F_{stat} = 1.661$, p = 0.2, df = 2).

a.



b.



c.

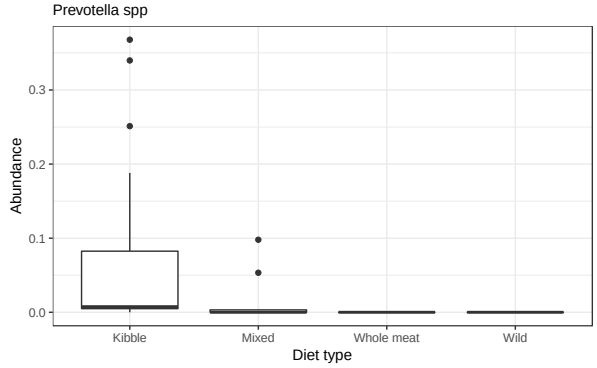


Figure 5. Relative abundance plots of bacterial ASVs that differed among diet types found by performing differential abundance analysis. **a.** Red wolves that consumed kibble diets had a higher relative abundance of the bacterial ASV *Cantenibacterium mitsuokai* versus individuals consuming the mixed, whole meat and wild diet type. **b.** Red wolves that consumed kibble diets had a higher relative abundance of the bacterial ASV *Holdemanella spp.* versus individuals consuming the mixed, whole meat and wild diet type. **c.** Red wolves that consumed kibble diets had a higher relative abundance of the bacterial ASV *Prevotella spp.* versus individuals consuming the mixed, whole meat and wild diet type.

Gut bacterial community structure was significantly different among FCS (Figure 6, PERMANOVA: Jaccard Psuedo-F = 1.7107, df = 2, R2 = 5.9%, p = 0.001; Bray Psuedo-F = 1.7821, df = 2, R2 = 6%, p = 0.018; unweighted Unifrac Psuedo-F = 2.3236, df = 2, R2 = 7.5%, p = 0.001). The change was explicitly between FSC 0 and FCS 2 (pairwise $p < 0.05$ for Jaccard and unweighted Unifrac distance). There was similar dispersion in the gut bacterial community among FCS (PERMDISP: Jaccard p = 0.2982, Bray-Curtis p = 0.2806, unweighted Unifrac p = 0.685). There were no correlations between community structure dissimilarity and stool consistency dissimilarity matrices (Mantel test: Jaccard, p = 0.109, $R^2 = 0.055$; Unifrac, p = 0.558, $R^2 = -0.015$; Bray p = 0.24, $R^2 = 0.02$).

There were no significant ASVs that were identified by differential abundance analysis. However, relative abundance of bacterial phylum *Bacteroidetes* was higher in FCS of 0 and FCS of 1 versus FCS of 2 (*DA.pea* p = 0.01; *DA.qpo* p = 0.02; *DA.spe* p = 0.006) (Figure 7).

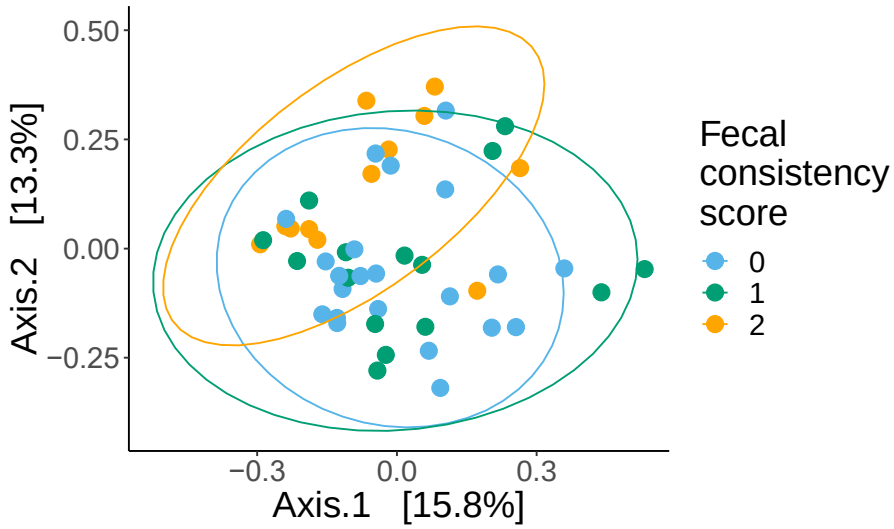


Figure 6 Principal Coordinate Analysis (PCoA) of fecal bacterial community structure from 49 red wolves (Unifrac distance). While there was overlap between each FCS, the gut microbiome composition was different among FCS. Specifically, red wolves with a FCS of 0 had a different gut microbiome composition versus red wolves with a FCS of 2. 95% confidence ellipses shown for FCS that have greater than 2 samples.

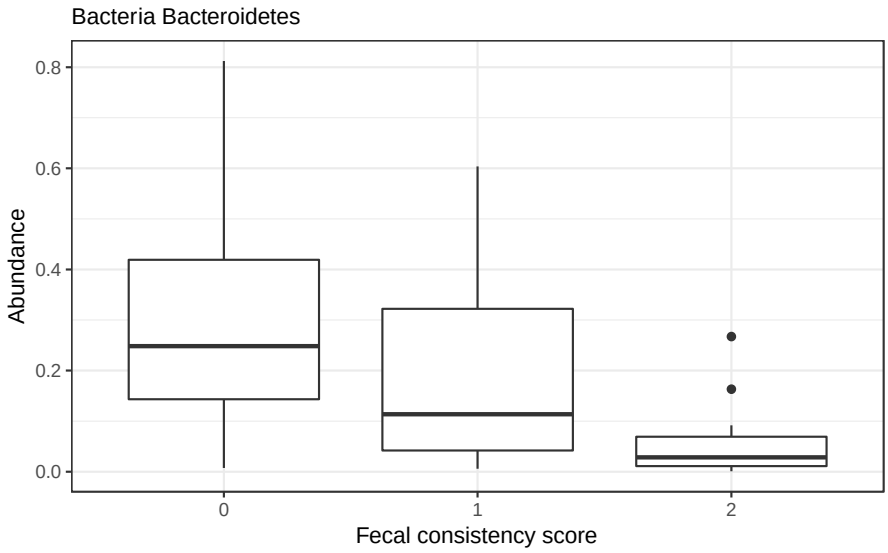


Figure 7 Relative abundance plots of bacterial ASVs that differed among FCS found by performing differential abundance analysis. Red wolves that have a FCS of 0 had a higher relative abundance of the bacterial phylum *Bacteroidetes* versus individuals with FCS of 1 and 2.

DISCUSSION

This study was the first to characterize the gut microbiome composition of the red wolf, a critically endangered canid, and its relationship with diet and FCS. I found that there was a link between gut microbiome composition and diet type. Additionally, I found an increase in the relative abundance of the bacterial taxa *Cantenibacterium mistsuokai, Holdmanella spp. and Prevotella spp.* in fecal samples of wolves fed a kibble diet compared to samples from wolves fed any of the other diet types (mixed, whole meat, wild). I found that the gut microbiome community differed between wolves with a FCS of 0 versus wolves with a FCS of 2. Lastly, I documented an increase in relative abundance of the bacterial phylum Bacteroidetes in wolves with a FCS of 0 versus a FCS of 2.

In the present study, the phyla present and their relative abundance in the gut microbiome of red wolves observed were similar to those observed in other canid species[2,16,61]. The gut microbiome of captive adult red wolf with a FCS 0, an animal presumed to be healthy, predominately consisted of Firmicutes (43.1%), Bacteroidetes (28.4%) and Fusobacteria (25.7%) with smaller percentages of Proteobacteria (1.9%) and Actinobacteria (0.8%). These results are consistent with previous findings that Firmicutes, Bacteroidetes, Fusobacteria and Proteobacteria are the most abundant phylum in the healthy canid gut microbiome[56]. Alessandri et al[2] found similar composition within the core microbiome of the domestic dog, Bacteroidetes (33.68%), Fusobacteria (25.53%) and Firmicutes (23.56%) with smaller percentages of Proteobacteria (6.29%),

Actinobacteria (0.93%). Additionally, Zhang and Chen[72] found similar composition within the gut microbiome of wild healthy adult grey wolf in China; Firmicutes (60%), Bacteroidetes (16.9%), Fusobacteria (9.2%), Proteobacteria (9.2%), Actinobacteria (4.6%).

The relative abundance of the top phyla found in the red wolf gut falls in the middle between the relative abundance of the same phylum in the gut microbiome of the domestic dog and the wild grey wolf in China. For example, a healthy red wolf gut is comprised of approximately 43% Firmicutes while the gut of a healthy domestic dog contains approximately 24% Firmicutes and the healthy grey wolf gut contains approximately 60% Firmicutes. This pattern also is consistent with the other four bacterial phyla. Median gut microbiota relative abundance in the red wolf could be caused by individual variation or differences in extraction methods. The methodology used in the present study was similar to Alessandri et al[2] but differed from Zhang and Chen[72] whom placed fecal samples in buffer for storage, used a different extraction method and older sequencing technology. The variations in methodology can lead to discrepancies in the sequence data produced. However, these results are not unreasonable given that the red wolf is a canid species largely being fed a domestic dog diet in captivity. It is plausible that the red wolf gut microbiome would be more similar to the domestic dog gut microbiome, based on the common diet of kibble, versus the grey wolf.

Changes in gut microbiota composition were seen in red wolves with a higher FCS, assumed to be unhealthy, when compared to red wolves with a FCS of 0, presumed to be healthy. In the present study, there was an increase in the relative abundance of Firmicutes and a decrease in the relative abundance of Bacteroidetes in red wolves with a FCS of 2, when compared to red wolves with an FCS of 0.A relative decrease in Firmicutes and Bacteroidetes is linked to IBD in humans[38] and canids[39]. Xenoulis et al[69] also saw a similar pattern in domestic dogs when comparing the duodenal mucosa microbiome in individuals with IBD to healthy individuals. However, other studies using fecal samples to investigate microbiome shifts associated with IBD recorded a decrease in Firmicutes and Bacteroidetes in the gut microbiome of domestic dogs who suffered from IBD compared to control dogs[39,57]. The present study and past literature using fecal samples all record a decrease in Bacteroidetes but differ on the relative abundance in Firmicutes present[39,57].

Species differences could cause the dissimilarity seen in gut microbiome community shifts between the present results and other studies. Although the domestic dog and red wolf are closely related, the event of domestication of dogs from the grey wolf happened approximately 15,000 to 12,500 years ago[18]. The domestic dog had much longer to adapt their microbiome to ‘captive’ conditions than the red wolf, which has only been in captivity for approximately 40 years. More importantlh, we do not want their microbiome adjusting to a captive diet as the goal is to eventually restart reintroductions of red wolves into their historic

ranges[27]. The red wolf natural gut microbiome should be kept intact to protect the species from opportunistic pathogenic bacteria that could be present in the natural landscape[64], and to ensure proper nutrient transformation, vitamin production and communication between the microbiota and the immune system[64].

While all microbiota in the gut are important, the phyla Firmicutes and Bacteroidetes are notable due to their involvement in the production of short chain fatty acids (SCFA). SCFA are produced when bacteria breakdown non-digestible dietary carbohydrates[22] and are necessary to generate vitamin B and K[22], energy for microbial metabolism[44] and impede growth of likely pathogens[22]. For the most part, Firmicutes produce butyrate, a SCFA that can suppress inflammation in immune and intestinal epithelial cells, encourage various properties that benefit intestinal barrier function[11,22] and stop the build-up of toxic metabolic waste products[32]. I found an increase in the relative abundance in Firmicutes between FCS 2 and FCS 0 fecal samples. Additionally, I found an increase in Firmicutes in the samples from kibble and mixed diet types compared to the whole meat diet type. The increase in relative abundance in Firmicutes seen in the present study could be due to the amount of starch present in the kibble diet, the most common diet type in this study. Firmicutes have been associated with a high carbohydrate diet[25]. The by-products of non-digestible carbohydrate fermentation by Bacteroidetes are acetate and propionate[50]. Acetate is the most common SCFA in the gut and the main component that

allows certain bacteria to kill pathogens within the GI tract[50]. Propionate provides energy, maintains homeostasis in the GI tract and monitors immune function[38]. I found an decrease in the phylum Bacteroidetes between a FCS 0 and FCS 2. This decrease in Bacteroidetes is commonly seen in dogs suffering from IBD versus healthy dogs[16]. This phylum has been documented as a beneficial taxon that supports GI tract health[69]. The presence of SCFAs are necessary to maintain GI health and as alterations in the relative abundance of the gut microbial community occur, the presence or concentration of SCFAs can change[15].

It is well known that diet type can influence the gut microbial community structure[16] and my results support this, showing difference in gut microbiome composition among the four diet types of the red wolf. The red wolf gut microbiome composition of the kibble and mixed diet type was dominated by Firmicutes while the whole meat diet gut microbiome was largely comprised of Fusobacteria. The wild wolves gut microbiome was almost equally inhabited by Firmicutes and Fusobacteria, with Firmicutes having slightly higher relative abundance. The relative abundance of Bacteroidetes was quite low in the wild wolves in comparison to the captive wolves on the three other diet types. Fusobacteria is associated with a high protein diet whereas an increase in Firmicutes, specifically the class *Erysipelotrichia,* is associated with a high carbohydrate diet[25]. There is a gap in the literature about the relationship between Fusobacteria and a high protein diet in canines[24]. It is reasonable that

the high carbohydrate diet is linked to the increase in Firmicutes, specifically the class *Erysipelotrichia,* as this bacterial class is positively correlated with markers for carbohydrate digestion and negatively associated with markers for protein digestion[6]. Still, the exact role and function of this class of bacteria is not fully understood[6]. For example, in humans, *Erysipelotrichia* is correlated to intestinal inflammatory diseases but in domestic dogs it is correlated with a healthy gut microbiome[6]. The kibble diet fed to red wolves is comprised of a high amount of starch in comparison to the wild diet of a red wolf[27], which could account for the increased abundance of Firmicutes in the feces compared to wolves on the whole meat diet. Alternative variables like digestibility, intake of substrate and the type of substrate that gets into the colon can influence the microbial community structure[57].

I recorded specific bacterial taxa that were differentially abundant among diet types. There was a relative increase of bacterial taxa *Cantenibacterium mitsuokai, Holdemanella spp* and *Prevotella spp.* in feces from wolves fed a kibble diet versus all other diet types. *Cantenibacterium mitsuokai* is an obligate anaerobe that has a positive relationship with starch break down and can generate acid from glucose, fructose, lactose and galactose[34]. Similarly, *Holdemanella spp.* are strict anaerobes that ferment sugars, the simple form of carbohydrates[14]. Moreover, *Prevotella spp.* are associated with fermentation and usage of polysaccharides, or carbohydrates bonded by various sugar molecules[55]. Knowing their relationship with carbohydrates, it is not surprising to

find an increase in relative abundance of these taxa in the gut of kibble diet fed wolves.

I found that diet type and FCS were not associated to each other, which could indicate that the underlying genetics of the red wolf can be playing a greater role than diet in the etiology of gut inflammation in this species. The kibble based captive diet contains large amount of imitation starch, which introduces domestic dog-like microbial species and shifts in the microbial community away from that of the wild red wolf. This shift in the nutritional composition of the diet could cause an increase or decrease in TLR5 activation at inappropriate times, leading to inflammation in the GI tract[27]. However, the lack of an association between diet type and FCS is more likely due to the fact that FCS may not be the best proxy for GI health in red wolves. While soft stool is a sign of IBD, it is not a unique symptom[33]. Diarrhea is a common health issue in canids[21] and can be caused by various factors including ingestion of inappropriate food items[21], appearance of a specific pathogen[58], antibiotics[21], a parasitic disease[12], intestinal tumors or mechanical obstruction[12]. Additionally, the presence of diarrhea can be sporadic in canids[21], making it an inconsistent measurement of GI health status. FCS is one component of a published IBD activity index for domestic dogs[33]. The published activity index also includes categories like change in weight loss, attitude, appetite, vomiting and stool frequency along with FCS[33]. Utilizing the entire canine IBD activity index may be a better proxy for IBD activity in the red wolf. Nevertheless, longitudinal data

should be collected in regard to FCS or the entire canine IBD activity index for each individual to give a more detailed picture of the GI health status. Additionally, analysis of a single fecal samples from each wolf only provides a single snapshot in time. Instead fecal samples should be collected over time to have a more inclusive observation of the flucturations, or lack thereof, in GI microbiota community within each wolf. Furthermore, there was a small sample size for the whole meat and wild diet type categories. The study would benefit from an increase in the number of wolves within those diet types.

There are still many unknowns about the relationship between IBD and the gut microbiota of the captive red wolf. Further research should be done to characterize the microbiota present in the duodenal mucosa of healthy red wolves and diseased red wolves to identify any patterns linked to IBD. It seems that the duodenum is where much of the inflammation is taking place for canids, specifically in red wolves[68]. Additionally, future studies should use methods to investigate the metabolism of the phyla Firmicutes, Fusobacteria and Bacteroidetes in the duodenal mucosa of a captive red wolf suffering from IBD. This will increase understanding of the presence and concentration of SCFA in the duodenal mucosa of diseased red wolves, unveiling more detailed information about the interaction between SCFA and the immune system. This can be done using gas chromatography to identify the molar concentration of SCFA from mucosal swabs. However, conducting these studies are challenging

in endangered species due to the availability and invasiveness of sample collection.

In conclusion, this study analyzed fecal samples to characterize the gut microbiota of the captive red wolf, a critically endangered canid that is suffering greatly from GI health issues such as IBD. I uncovered a higher relative abundance of the bacterial phylum *Bacteroidetes* in captive wolves compared to the two wild wolves. Considering that IBD has not been described in the wild population[27], this phylum could be important in the pathogenesis of IBD in the red wolf as it is more abundant in captive animals than wild wolves. Additionally, I found an increased relative abundance of *Bacteroidetes* and a decreased relative abundance of *Firmicutes* in red wolves with a FCS of 0 compared to red wolves with a FCS of 2. Lastly, I documented an increase in relative abundance of specific bacterial taxa, *Cantenibacterium mitsuokai, Holdemanella spp.* and *Prevotella spp.,* in red wolves fed a kibble diet versus all other diet types. Using proper techniques, detailing the gut microbiota present can give insight into the status of gut health and function in individuals. With further research and understanding, microbes in the form of probiotics could eventually aid in suppressing or reversing IBD symptoms in the captive red wolf. There are approximately 280 red wolves left in the world, with 90% of the individuals living in captivity. While this *ex situ* population is necessary for the continued existence of the species, the captive environment brings manipulated diets and man-made structures to live in[67]. These factors, along with others, can cause shifts in the gut

microbiome[67], negatively impacting GI health of the red wolf. It is essential for the captive population to be healthy to facilitate reintroducing these animals to their natural landscape in the future.

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BIOGRAPHY

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