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THE GUT MICROBIOTA OF A WILD AMERICAN BLACK BEAR (Ursus americanus) POPULATION

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THE GUT MICROBIOTA OF A WILD AMERICAN BLACK BEAR (*Ursus americanus*) POPULATION

By

Sierra J. Gillman

THESIS

Submitted to
Northern Michigan University
In partial fulfillment of the requirements
For the degree of

MASTERS OF SCIENCE

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March 2020
THE GUT MICROBIOTA OF A WILD AMERICAN BLACK BEAR (*Ursus americanus*) POPULATION

This thesis by Sierra J. Gillman is recommended for approval by the student’s Thesis Committee and Department Head in the Department of Biology and by the Dean of Graduate Education and Research.

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Dr. Lisa Schade Eckert, PhD
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ABSTRACT

THE GUT MICROBIOTA OF A WILD AMERICAN BLACK BEAR (*Ursus americanus*) POPULATION

By

Sierra J. Gillman

The gut microbiome (GMB), the mutualistic microbial communities located in the gastrointestinal tract (GIT), has co-evolved in vertebrates to perform micro-ecosystem services to facilitate physiological functions. Despite the key role of the GMB in host health, wildlife managers have been slow to consider the ramifications of anthropogenic pressures to wildlife-GMB diversity. For example, although diet is one of the most influential determinants of a host’s GMB, many wildlife agencies allow baiting with human-provisioned foods to facilitate the harvest of wildlife such as American black bear (*Ursus americanus*). Additionally, much of our knowledge of wildlife-GMB relationships is based on studies of colon GMB communities derived from the feces of captive specimens. To better understand wildlife-GMB relationships, I first aimed to characterize wild black bear GMB communities in the colon and jejunum, two functionally distinct regions of the gastrointestinal tract (GIT). Second, I estimated the proportional contribution of human-provisioned foods to the diets of black bear and evaluated the effect of human-provisioned foods on the GMB at each GIT site. I engaged hunters as citizen scientists to collect biological samples from legally harvested black bears, 16S rRNA gene amplicon sequencing to identify microbial taxa, and stable isotope analysis of black bear hair to estimate diet. My results suggest that the jejunum and colon of black bears do not harbor significantly different GMB communities, but that increased proportions of human-provisioned foods in black bear diet, specifically corn, and significantly reduces GMB diversity.
DEDICATIONS

I cannot fully put into words my immense respect, appreciation, and gratitude for my thesis and research advisor, Dr. Diana Lafferty. You have guided and supported me over these last two years, and are an example of excellence as a researcher, mentor, instructor, and role model. I am eternally grateful for having had the opportunity to learn from you.

You have been instrumental in molding me into the scientist I continue to become.

To my family, the Carey Clan, thank you for supporting me throughout my life as I have pursued my love for wildlife. Each of you have been there during my failures and celebrated my successes. Dad & Mom, from an early age you allowed me to follow my passions, and I am who I am today because of you.

I thank with love and appreciation my supportive and endlessly patient husband, Tom; without you I would never have gotten this far. Thank you for not only your willingness to join me on my academic journey but your encouragement when I most need it. I can always depend on you to be by my side.

Finally, to Barley my four-legged friend, you were there for Tom when I couldn’t be. You made me get out of the house for some fresh air when I didn’t want to. You provide a much needed example of how to relax in life, and you have been a great comfort every day.
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INTRODUCTION

1.1 The gut microbiome

Due to advances in technologies such as next generation sequencing, scientists are now able to analyze unculturable microorganism communities. Through recent investigations of these previously unknown microbes, scientists discovered that mammals are metagenomic, composed of both their genes and the collective genome of their co-evolved and interdependent microbial communities, their microbiome (McFall-Ngai et al. 2013). The distal gut is home to the vast majority of mammalian microbial communities (Bäckhed et al. 2005), the dynamic and influential gut microbiome (GMB), which is intimately linked to mammalian health, fitness, and adaption. Indeed, the GMB promotes and facilitates countless physiological functions in mammalian hosts including immune system maintenance, tissue development, behavior, digestion, and vitamin synthesis (Hill 1997, Hooper et al. 2012, Nicholson et al. 2012, Foster and McVey Neufeld 2013).

Myriad factors shape the mammalian-GMB and include sex, life stage, diet, and the external environment of the host (Muegge et al. 2011, Amato et al. 2013, McKenney et al. 2015, Dominianni et al. 2015). In wildlife, the GMB composition is dependent upon host’s habitat quality and consequently food availability and may act as a mechanism for plasticity, enabling hosts to acclimate to a changing environment brought about by anthropogenic pressures, resulting in long-term implications for wildlife-host survival and evolution (Barelli et al. 2015, Hauffe and Barelli 2019). In a time of increased anthropogenic pressures on wildlife due to unfettered habitat destruction, loss of natural prey/foods, and rapid urbanization, the GMB could prove to be a valuable tool in wildlife
management and population health monitoring initiatives. Yet, because of the complex inter-relationship between mammals and their GMB, the number of studies addressing the role of the GMB on mammalian health is almost exclusively limited to human studies and model organisms (e.g., lab rodents). Additionally, although the implications of perturbations of the GMB on wildlife conservation are numerous, only a few have been addressed (Amato 2013, Cheng et al. 2015, Barelli et al. 2015, Bahrndorff et al. 2016, Trevelline et al. 2019). As such, investigating the importance of the GMB of wildlife species and the impact the GMB has on wildlife health is crucial.

1.2 Ecology of black bears

Black bears (Ursus americanus) are charismatic, and ecologically important large carnivores with the adaptability to live in a variety of ecosystems. The total black bear range in the United States is 3.5 million km², representing 45-60% of their historical range (Scheick and McCown 2014). Black bears are living in closer proximity to humans due to increased habitat fragmentation caused by urbanization across North America, resulting in increased human-wildlife conflict (Don Carlos et al. 2009, Greenleaf et al. 2009). Although physiologically a carnivore due to their digestive physiology (i.e., short, simplistic gastrointestinal tract), black bears are opportunistic omnivores, often serving as important seed dispersers (Stevens and Hume 1995, Enders and Vander Wall 2012). Black bears also have complex feeding phases during each year (Nelson et al. 1983).

In the Upper Peninsula of Michigan, the black bear population is estimated to be between 8,000–11,000, and over 1,000 black bears are legally harvested in the Upper Peninsula annually, providing an opportunistic occasion to engage hunters as citizen scientists to collect biological samples from regions of the GIT that would otherwise
require invasive collection. In addition, Michigan is one of 12 states that allows the use of human-previsioned foods to facilitate black bear hunting. Thus, as a common carnivore with a broad diet and populations widely distributed and harvested across much of North America, black bears are an excellent model for investigating the carnivore-GMB relationship and to determine the GMB response to human-provisioned foods in a wild carnivore’s diet.

1.3 Research overview

As large carnivores suffer the largest range contractions of all terrestrial mammals due to anthropogenic pressures (Ripple et al. 2014), understanding what influences carnivore health and fitness could improve conservation and management initiatives world-wide. Investigating the carnivore-GMB with methods used to research the human GMB could be applied to meet conservation challenges such as monitoring habitat quality, adaptative capabilities via metabolic expression of GMB, and the health risks of human-provisioned foods to diverse wildlife across an increasingly humanized world. In my first chapter, I characterize and compare GMB diversity of the jejunum (previously unstudied in a wild carnivore population) and the colon of legally harvested American black bear through 16s rRNA gene amplicon sequencing and through the engagement of hunters as citizen scientists. In my second chapter, I used stable isotope analysis to analyze the proportional contribution of human-provisioned foods to black bear diet in the Upper Peninsula of Michigan and I examined the effect of human-provisioned foods on GMB diversity in black bears.
1.1 Introduction

1.1.1 Background

Over the last century, humankind has appreciably altered Earth’s ecosystems in myriad ways. Anthropogenic changes such as over exploitation of natural resources (Schipper et al. 2008, Gutti et al. 2012), urbanization (Lewis et al. 2015, Šálek et al. 2015), pollution, and human-mediated climate change (Descamps et al. 2017) have caused irrevocable biodiversity loss, with large carnivores suffering the greatest population declines and range contractions of all terrestrial mammals worldwide (Ripple et al. 2014). For example, six of the eight extant bear species are considered vulnerable to extinction (Wiig et al. 2015, Dharaiya et al. 2016, Garshelis and Steinmetz 2016, Scotson et al. 2017, Swaisgood et al. 2017, Velez-Liendo and García-Rangel 2018). Whether it be giant pandas (*Ailuropoda melanoleuca*) suffering as a result of severe habitat fragmentation (Loucks et al. 2001, Liu 2001), or polar bears (*Ursus maritimus*) facing reduced foraging opportunities due to rapidly retreating summer sea ice (Derocher et al. 2013, Rode et al. 2014), or Asiatic black bears (*Ursus thibetanus*) subject to high levels of poaching for commercial trade of bear body parts (e.g., bear bile; Liu et al. 2011), bears in particular are poignant examples of the hurdles ecologist and conservationist face in the struggle to save ecologically and culturally important carnivores around the world.
While the threats carnivores face in their degraded external environments are relatively well-known (e.g., depleted prey [Wolf and Ripple 2016], genetic isolation [Randi 1993, McRae et al. 2005, Dixon et al. 2007]); the threats carnivores face from changes in their own internal environments, as a consequence of human-mediated environmental perturbations, are almost entirely unknown. For example, how do changes in carnivore gut microbiota, the consortia of microorganisms (i.e., bacteria, fungi, viruses) within the gastrointestinal tract (GIT), affect host fitness? In fact, the intimate co-evolution between vertebrate hosts and their gut microbiome (GMB) is an emerging area of interest in wildlife ecology. Studies show GMBs perform countless micro-ecosystem services for their hosts (McKenney et al. 2018a), facilitating critical physiological processes such as digestion (Nicholson et al. 2012) and vitamin synthesis (Hill 1997), immune system maintenance (Hooper et al. 2012) as well as host weight regulation (Turnbaugh et al. 2009). Indeed, research provides strong evidence of intraspecific variation and heritability in GMBs, suggesting GMBs may affect host phenotype and ultimately host’s adaptive potential (see review by Hauffe and Barelli 2019).

Though intrinsic and extrinsic factors influencing GMBs are multifaceted (e.g., host’s external environment [Amato et al. 2013], sex [Dominiani et al. 2015], life stage [McKenney et al. 2015], phylogeny [Ley et al. 2008, Phillips et al. 2012], and diet [David et al. 2014]), human-mediated shifts in GMB community composition may lead to altered micro-ecosystem function, affecting nutrient uptake and host health (Hooper et al. 2002, Turnbaugh et al. 2006). Specifically, as GMB composition is dependent particularly upon host’s habitat and consequently food availability and therefore diet, the GMB may act as
a mechanism for plasticity, enabling hosts to acclimate to a changing environment brought about by anthropogenic pressures and resulting in long-term implications for host survival and evolution. Further, due to the sensitivity of the GMB to host habitat, the composition and functional profile of wildlife GMB could serve as a proxy for habitat quality, and could therefore prove to be a valuable tool in wildlife management and monitoring initiatives (Barelli et al. 2015).

Much of our knowledge of wildlife-GMB relationships is based on studies of captive specimens (Clauss et al. 2009, McKenney et al. 2018b, Hale et al. 2018), due in part to the elusive nature of many wild species. However, recent studies revealed captivity can alter GMB community composition (Cheng et al. 2015, Clayton et al. 2016, Borbón-García et al. 2017, McKenzie et al. 2017). Further, most wildlife-GMB research has focused on colon samples (i.e., feces), where fiber fermentation occurs, understandably because collecting samples from other regions of the GIT is highly invasive. However, within omnivores and carnivores nearly 90% of fats, carbohydrates, and proteins are absorbed in the jejunum, the middle section of the small intestine (Borgström et al. 1957, Thomson et al. 2003). Thus, by focusing research attention on microbial communities associated with the colon, scientists are unable to fully understand important evolutionary relationships between wildlife and their GMBs in other functionally distinct regions of the GIT, relationships that may be important when considering wildlife management and conservation initiatives. Further, incorporating analyses of GMB community composition and structure into ecological research initiatives may aid our understanding of wildlife-GMB co-evolution and provide novel insights into host health (Amato 2013).
The American black bear (*Ursus americanus*) is both an ecologically and culturally important large carnivore with a unique life history. Black bears physiologically retain a simple GIT characteristic of carnivores (i.e., lack of cecum, short GIT length; Stevens and Hume 1995), but consume an omnivorous diet, and exhibit substantial among-individual dietary variation (Lafferty et al. 2015) and behavioral plasticity (Ayres et al. 1986, Baruch-Mordo et al. 2014). Beyond serving as both predators and prey, black bears function as important seed dispersers (Willson 1993, Enders and Vander Wall 2012, Harrer and Levi 2018) due to their extremely rapid digestion time (Pritchard and Robbins 1990). Black bears also exhibit four physiological stages, the most complex of all carnivores (Nelson et al. 1983). In winter months, black bears undergo torpor (i.e., Stage I), characterized by fasting and inactivity when food resources are scarce. Upon emergence from the den, black bears enter walking hibernation (i.e., Stage II), an anorectic phase during which black bears do not eat for the initial 10 to 14 days after den emergence. Black bears then resume normal activity (i.e., Stage III), eating and drinking at will. During fall months, black bears enter hyperphagia (i.e., Stage IV), a period of increased caloric intake mediated by changes in seasonal digestive ability (Brody and Pelton 1988) in which they can gain up to 1kg daily (Hellgren 1988), which is essential for surviving Stage I. While length of torpor varies by latitude and weather conditions, torpor can last up to seven months (Fowler et al. 2019), during which time black bears typically do not eat. Additionally, black bears have low reproductive rates due to slow maturation rate and long-term maternal care and relatively small litters (two to four cubs; Samson and Huot 1995) that are dependent upon fat reserves acquired during hyperphagia (Samson and Huot 1995). Moreover, although
black bears are one of two bear species listed as least concern on the IUCN Red List of Threatened Species (Garshelis et al. 2016), black bear geographic range is increasingly fragmented due to urbanization across North America. With diminishing habitat, black bears are living in closer proximity to humans, resulting in increased human-wildlife conflict (Don Carlos et al. 2009, Greenleaf et al. 2009). Furthermore, thousands of black bears are legally harvested each year in the USA and Canada, providing an opportunistic occasion to train hunters as citizen scientists to collect biological samples from regions of the GIT that would otherwise require invasive collection. Thus, as a common carnivore with a broad diet and populations widely distributed and harvested across much of North America, black bears are an excellent model for investigating carnivore-GMB relationships.

1.1.2 Objectives

In the current study, I aimed to characterize and compare black bear GMB communities associated with two functionally distinct regions of the GIT, the jejunum and colon. Based on previous GMB research on omnivores (Hayashi 2005, Yasuda et al. 2015, Sundin et al. 2017, Xiao et al. 2018), I hypothesized that the jejunum and colon would harbor distinct GM community structures (e.g., evenness, richness, phylogenetics). To my knowledge, my research is the first investigation of GMBs associated with operationally distinct regions of the GIT in a wild carnivore population.
1.2 Methods

1.2.1 Study area and sample collection

I received an exemption from review by the NMU Institutional Animal Care and Use Committee because samples were collected opportunistically from dead bears that were legally harvested by hunters not involved with my study. I collected samples with permission from individual hunters/guides under a Michigan DNR – Wildlife Division – Scientific Collector's Permit (#SC 1613).

I sampled black bears across the Upper Peninsula (UP) of Michigan, USA (7°00’ – 45°09’N, 90°18’ – 84°37’W). The UP consists of primarily deciduous hardwood forest with intermittent conifer swamps, wetlands, shrub patches, and agriculture with elevation ranging between 170 m to 600 m above sea level. Average daily temperatures during the September-October sampling frame varied from a low of -1.6°C to a high of 22°C.

I collected colon and jejunum content from legally harvested black bear and one roadkill bear (n=35) within 30 minutes of death during the annual fall harvest season (September 10 to October 26, 2018). Colon contents were collected from deceased bears with a sterile tongue depressor and immediately placed in sterile, 15 mL centrifuge tubes containing 95% ethanol. To collect jejunum contents, bear abdomens were opened, and the stomach and intestines were removed. Hunters incised the small intestine 16 inches below the pyloric sphincter and poured jejunum content (from the intestines above the incision) into sterile 15 mL centrifuge tubes containing 95% ethanol (see Appendix E: Supplemental 1 for hunter instructions). All samples were stored at room temperature until microbial DNA was extracted (~ 50 days). Sex was recorded and Michigan Department of Natural Resources provided age data from teeth they collected per their
harvest registration protocols (Table 1.1). Bears were then categorized into age classes (i.e., yearling=1, subadult=2–3, adult ≥ 4).

1.2.2 DNA isolation and sequencing

A total of 66 samples were collected from black bears (32 jejunum, 34 colon; see Table 1.1). I extracted microbial DNA from jejunum and colon samples using DNeasy PowerSoil Kits (QIAGEN, Hilden, Germany), following manufacturer’s protocol with the addition of (1) a heat-step of 10 minutes at 65°C at the beginning of the protocol to breakdown proteins and (2) a second elution as the final step of extraction (see Appendix F: Supplemental 2), as previously described by McKenney et al. 2017. I quantified DNA yields using a NanoDrop 2000c (ThermoFischer Scientific, Massachusetts, USA) and stored extractions at -20°C. After all extractions were complete, standardized DNA aliquots were shipped to Argonne National Laboratory (Lemont, IL, USA) for PCR amplification and paired-end DNA sequencing of the 16S rRNA V4 gene region, according to methods described by (Caporaso et al. 2012).

1.2.3 Bioinformatic analysis

Multiplexed EMP-paired-end sequence reads were imported into Quantitative Insights Into Microbial Ecology (QIIME2, version 2019.4; Bolyen et al. 2019) and demultiplexed. Sequences were joined, denoised, filtered to remove chimeras and residual Phix reads, and dereplicated; amplicon sequence variants (ASVs) were called using DADA2 QIIME2 plugin (Callahan et al. 2016) and sequence lengths were truncated to 150bp.

1.2.4 Taxonomic classification

I used the SILVA 99 database for V4 region (version 132; Quast et al. 2012) to assign taxonomic classification in QIIME2, using a trained Naïve Bayes sklearn classifier
(Bokulich et al. 2018) to classify organisms at the genus level. Sequences were aligned with MAFFT (Katoh and Standley 2013), a plugin for phylogenetic diversity analysis, which removes highly variable positions in the process. I further filtered samples to remove chloroplast, mitochondrial, and Archaea sequences, as well as unidentified microbial DNA unidentified below kingdom level, and any unassigned sequences (See Appendix G: Supplemental 3 for QIIME2 pipeline).

1.2.5 Statistical analysis

All statistical analyses and visualizations were conducted using R (version 3.6.; R. Core Team 2018) and Rstudio (version 1.2.1335; Allaire 2012) as outlined in Appendix H: Supplemental 4 for R script. Data were imported into R for downstream analysis using qiime2R (R, version 0.99.12; Bisanz 2018) and converted to phyloseq (version 1.28.0; McMurdie and Holmes 2013) objects. I rarefied ASVs at a depth of 1050, 65 samples, and 68,250 total sequences (3.7% of the original input), with an average frequency of 32,508 reads per samples (n=65) for alpha and beta diversity analysis. I investigated diversity of GMB communities per GIT site through analysis of Shannon (Hill 1973) and inverse Simpson (Simpson 1949) diversity indices using microbiome (R, version 1.6.0; Lahti and Shetty 2012), and of Faith’s Phylogenetic Diversity (Faith 1992) via picante (R, version 1.8; Kembel et al. 2010). I performed linear mixed effects models (LMM) for analyses to determine the relationship between Faith’s phylogenetic diversity (PD) and GIT site using R packages lme4. As research on wild hyenas has shown that external body site surface microbiome communities can differ depending upon sex and age-class (Rojas et al. 2020), I used GIT site, sex and age-class (yearling =1, subadult=2–3, adult ≥ 4) as categorical fixed effects; alpha diversity indices were the response variables and
individual was included as a random effect. In each model, I checked residuals to confirm
model requirements (e.g., normality, homoscedasticity, residuals). To determine when
interactions between main effects should be considered, I fit models with the maximum
likelihood (ML) and compared likelihood ratio tests via the lme4 function, which
performs Wald Chi-squared tests for LMM. Final models were fit with restricted
maximum likelihood (REML; Table 1.2). I determined the significance of main effects
and interactions within the top models through car (version 30-6; Fox and Weisberg
2018) also using Wald Chi-squared tests. Samples with no known bear age (n=4) were
dropped from LMM analysis and all other samples were retained (n=61). Faith’s PD and
inverse Simpson diversity values were log-transformed prior to analysis, due to their
skewed values. If main effects or interactions were significant, I acquired estimated
marginal means (EMMs) of pairwise comparisons for post hoc testing with Tukey
adjustment with emmeans (version 1.4.1; Lenth et al. 2018).

For beta diversity, I quantified compositional dissimilarity between GIT sites, sex,
and age-class using quantitative non-phylogenetic Bray-Curtis distance matrix (Bray and
Curtis 1957) using the vegdist and metaMDS functions in vegan (R, version 2.5-5) on
rarified data. I subsequently visualized Bray-Curtis results via non-metric
multidimensional scaling (NMDS) using ggplot2 (R, version 3.2.1; Wickham 2016), and
created heat trees using metacoder (version 0.3.3; Foster et al. 2017) and taxa (version
0.3.2; Foster et al. 2018). I also calculated the quantitative phylogenetic weighted Unifrac
distance (Lozupone and Knight 2005) using the phyloseq function Unifrac and plotted
these data on principle coordination analysis (PCoA) ordination plots. I performed
permutational multivariate analysis of variance (perMANOVA; Anderson 2001) on each
distance matrix using the *vegan adonis* function with a strata for subject ID. I performed an analysis of multivariate homogeneity (PERMDISP; Anderson 2006), an analog of Levene’s test for homogeneity of variance, again with a strata for subject ID, with *vegan betadisper* and *permutest* functions to test for significant differences in sample heterogeneity between GIT sites. Finally, I used Linear discrimination analysis Effect Size (LEfSe; Segata et al. 2011) in the Galaxy online tool (https://huttenhower.sph.harvard.edu/galaxy) to identify any ASVs differentially represented between GIT sites. I considered a $p$-value threshold of 0.05 significant for each test performed.

1.3 Results

1.3.1 Community composition of the GIT sites

I identified three major phyla (Firmicutes, Proteobacteria, Actinobacteria) within the jejunum and three major phyla (Firmicutes, Proteobacteria, and Epsilonbacteraeota) within the colon (Figure 1.1A). In addition to the three phyla identified in each GIT site, all minor taxa, representing <1% of the total abundance, were combined into a single category (Minor). I defined major taxa as representing >1% of ASVs. Firmicutes and Proteobacteria were the only major phyla detected in all samples, with Firmicutes being the most dominant phylum, constituting a mean of 71% ($\pm$ 34% SD) of the jejunum community and mean of 60% ($\pm$ 33% SD) of colon community, whereas Proteobacteria constituted a mean of 24% ($\pm$ 30% SD) of the jejunum community and 33% ($\pm$ 30% SD) of the colon community. Actinobacteria constituted 1.6% ($\pm$ 2.9% SD) within the
jejenum, and the phyla Epsilonbacteraeota constituted 5.4% (± 10% SD) of the colon community. All other phyla were minor (< 1% of ASVs) within both communities.

At the genus level, I identified 21 major taxa within the GIT. The jejunum harbored 13 major taxa, six of which were unique to the jejunum and seven were shared with the colon. The colon harbored 15 major taxa, eight of which were found solely within the colon (Figure 1.1B). Sarcina dominated the jejunum, making up a mean of 17% (± 33% SD) of identified genera, followed by Lactobacillus at 12% (± 26% SD), and Escherichia-Shigella at 12% (± 20% SD). Escherichia-Shigella dominated the colon community, constituting a mean of 27% (± 27% SD) of genera, followed by Sarcina at 15.8% (± 28% SD).

1.3.2 Community composition of age-classes

I identified five major phyla (Firmicutes, Proteobacteria, Epsilonbacteraeota, Actinobacteria, Tenericutes) in yearlings, three major phyla (Firmicutes, Proteobacteria, and Epsilonbacteraeota) for subadults, and two major phyla (Firmicutes, and Epsilonbacteraeota) in adult black bears (Figure 1.1C). Similar to the colon and jejunum GMB communities, Firmicutes and Proteobacteria were the only major phyla detected in all age-classes. For yearlings, Firmicutes were the most dominant phylum, constituting a mean of 54.4% (± 31% SD), whereas Proteobacteria constituted a mean of 36% (± 25% SD). The rest of the GMB of yearlings were made up of Epsilonbacteraeota 5.5% (± 14% SD), Actinobacteria 1.1% (± 2.6% SD), and the phylum Tenericutes 1.6 (± 5.8% SD), which was only found in yearlings. In subadults, Firmicutes made up 67% (± 32% SD), Proteobacteria made up 28% (± 28% SD), and Epsilonbacteraeota made up 3% (± 7% SD). The GMB of adults only consisted of Firmicutes 71% (± 37% SD) and
Proteobacteria 26% (± 236% SD). All other phyla were minor (< 1% of ASVs) within both communities.

At the genus level, I identified 25 major taxa within the three age-classes (Figure 1.1D). Yearlings harbored 15 major taxa, however, only two taxa were found solely in the GMB of yearlings, *Mycoplasma* 1.6 % (± 5.8% SD) and a genus found in the family Neisseriaceae 1% (± 3.9% SD). All other taxa found in the yearlings were either shared with either one or both subadults and adults. Further, although Actinobacteria and Tenericutes were major phyla of the yearling GMB, none were major at the genus level. Subadults had five unique taxa *Staphylococcus* 3.8% (± 1.9% SD), *Lactococcus* 3.5% (± 1.2% SD), *Leuconostoc* 1.5% (± 4.4% SD), *Bibersteinia* 1.1% (± 3.9% SD), and a bacteria found in the family Peprostreptococcacea 1.0% (± 2.2% SD). Subadults also had the largest number of minor taxa (n=280). Adult black bears had four unique taxa, *Bacillus* 4.9% (± 2.1% SD), *Sporosarcina* 2.4% (± 1.1% SD), *Moraxella* 2.2% (± 7.6% SD), and *Weissella* 1.7% (± 6.9% SD). The GMBs of yearling and subadult black bears were dominated by *Escherichia-Shigella* 30% (± 23% SD) and 17% (± 24% SD) respectively. The second most dominant genera of yearling black bears was *Clostridium sensu stricto 1* with 17% (± 26% SD), followed by *Sarcina* 15% (± 33% SD). *Sarcina* was the second most dominant genera in subadults 15% (± 30% SD), followed by *Lactobacillus* 12% (± 26% SD). *Sarcina* dominated the GMB of adults, making up a mean of 22% (± 33% SD) of identified genera, followed by *Escherichia-Shigella* at 18% (± 29% SD), and *Turicibacter* 9.6% (± 23% SD).
1.3.3 Alpha and beta diversity

Overall, the top LMM model for Faith’s PD diversity included no interactions (Table 1.2) and only age-class significantly influenced Faith’s PD ($\beta_{\text{sub}}=0.3$, $\beta_{\text{adu}}=-0.07$, $\chi^2=8.62$, $p=0.014$; Table 1.3). Contrasts of EMMs for age-class showed significant difference in Faith’s PD was between subadults and adults only ($p=0.05$; Table 1.4). I found no significant differences in either Shannon diversity or inverse Simpson diversity between GIT sites, sexes, or age-classes in the top LMM models (Table 1.3).

GIT site and age-class varied in their degree of dispersion for both Bray-Curtis and weighted UniFrac distance matrices (Tables 1.5, 1.6). However, PERMDISP results indicate a significant difference in homogeneity of dispersion between GIT sites (F=12.37, $p=0.002$) and age-class (F=5.26, $p=0.011$) for Bray-Curtis. Tukey honestly significant difference results between age-classes determined the significant differences in homogeneity were between yearlings and subadults $p=0.006$, and yearlings and adults $p=0.04$. Therefore, the significance of GIT site on GMB composition may be an artifact of within-group dispersion, as opposed to differences in centroid position. Moreover, ordination plots show greater variation in diversity among jejunum samples and by comparison, colon diversity is more conserved, however, I also observed substantial overlap in microbiome composition (Figure 1.2). In addition to GIT site and age-class, sex significantly influenced Weighted UniFrac GMB distances ($R^2=0.005$, $p=0.007$; Table 1.7); yet, there appeared to be no real clusters on PCoA plots (Figure 1.2).

1.3.4 Significantly enriched bacteria between GIT sites

I used logarithmic Linear Discriminate Analysis (LDA) score of 2.0 as the cutoff for LEfSe analysis (Segata et al. 2011) and found 23 ASVs differentially represented
between the jejunum and colon at genus level (LDA score ≥ 3.65, p<0.05; Table 1.7).
Within the jejunum, seven taxa were differentially abundant, none of which were major
taxa and none identified below the level of order in three phyla: Proteobacteria (n=4),
Actinobacteria (n=2), Firmicutes (n=1; Table 1.7).

Two of the unique major bacteria were differentially abundant in the colon
communities (Turicibacter 8.6% [± 19% SD], Helicobacter 5.3% [± 10% SD]), and one
unidentified taxa within the family Enterobacteriaceae 1.2% (± 3% SD) and two
unidentified taxa within the family Peptostreptococcaceae 1.2% (± 1.9% SD; Table 1.7).
Further, although found in both jejunum and colon, Escherichia-Shigella and Clostridium
sensu stricto I were differentially abundant in the colon microbial community, and nine
unidentified minor genera in the three major phyla (Firmicutes, Proteobacteria, and
Epsilonbacteraeota) were also differentially abundant in the colon. Although the colon
harbors approximately double the number of enriched bacteria, the majority are
unidentified taxa occurring across the taxonomic hierarchy of the three major phyla (e.g.,
family, order, class). Conversely, I found a higher degree of phylogenetic variability
among enriched bacteria in the jejunum compared to the colon (black stars; Figure 1.3).
Further still, GMB taxonomic structure from the kingdom level to genus in the jejunum
exhibit greater abundance of minor taxa contributing to overall GMB composition,
whereas the colon was dominated by fewer taxa primarily within the major phyla (Figure
1.3). LEfSe results revealed no significantly enriched bacteria between age-classes.
1.4 Discussion

1.4.1 The jejunum and colon do not harbor significantly different GMB communities

To my knowledge, my research is the first to implement hunters as citizen scientists to compare microbial communities in two operationally distinct regions of the GIT of a wild carnivore population as well between age-classes of American black bears. Similar to previous studies, black bear GMB membership and alpha diversity were influenced in part by age-class (Gomez et al. 2012, Yatsunenko et al. 2012, McKenney et al. 2015, Dominianni et al. 2015, Rojas et al. 2020), with subadults GMB harboring higher phylogenetic diversity compared to adults (Figure 1.4). I found the colon harbored double the number of significantly enriched bacteria (Table 1.7). Additionally, there was a higher abundance of minor taxa and phylogenetic branching contributing to jejunum GMB community composition (Figures 1.1, 1.3). Yet, despite perceived differences, alpha diversities, specifically Faith’s PD, did not differ between the two GIT sites. While there is evidence to suggest that Bray-Curtis distances were affected by GIT site and age-class (Tables 1.4, 1.5), these findings should be interpreted cautiously, due to the lack of distinction between perMANOVA and PERMDISP results. Further still, although weighted Unifrac was not heterogeneous for GIT site, age-class, or sex, PCoA plots show no real clustering or discernable pattern (Figure 1.2C), unlike previous studies that have investigated GMB community differences between body sites (Greene and McKenney 2018, Rojas et al. 2020). Given the different physiologies and micro-environments of the jejunum and colon, with fat, carbohydrates, and protein absorption occurring in the jejunum and fermentation predominantly occurring in the colon, the lack of clear differences within distance matrices clustering observed in the black bear microbial
diversity measures was unexpected, especially considering that differences in community structure and membership have been observed previously in other species with omnivorous diets.

I propose two potential explanations for my findings: 1) the generalist diet and rapid digestion time (digest time of 13 hours for meat/hair and 7 hours for foliage) of black bears, and overall simplicity of the gastrointestinal tract (i.e., short length and lack of vestigial organs such as appendix and cecum), may keep the jejunum and colon in a constant state of disturbance; or 2) perhaps black bears have evolved to meet their physiological needs through low GMB diversity linked to adiposity.

As generalist consumers with a simplistic GIT and rapid digestion, the climax community of black bear GMB could be dominated by bacteria typically considered pioneer species. Although bacterial colonization is prompt, rapid transit time from consumption to defecation could cause consistent shedding of microbial communities and/or, may prevent succession from progressing beyond a pioneer stage – because rapid transit time may favor generalist/opportunistic microbial species. For example, in macro-environments such as forests, short disturbance intervals can lead to communities dominated by early successional species, or pioneer species that can respond quickly to vacant niches (Grime 1977, Connell 1978). The macro-concept of succession can also be applied to microbial ecosystems. Further, although the appendix was once thought to be vestigial, researchers recently proposed the appendix functions as a reservoir for microbes (Bollinger et al. 2007, Smith et al. 2009), a concept corroborated by recent studies (Xiao et al. 2018, Greene and McKenney 2018). For example, Greene and McKenney (2018) opportunistically sampled GMBs from the appendix, cecum, and colon.
of deceased captive aye-aye and found appendices were the most distinct sampling location in both diversity and composition, whereas the cecum and colon were more homogenous. Although wildlife species with physiologically more complex GITs might harbor more stable microbiota communities, carnivores such as black bears possess neither a cecum nor appendix, thus lacking a potential microbial reservoir to shield microbial communities from constant disturbance associated with a simple GIT with rapid food passage transit times.

Alternatively, the role of black bear GMBs may be more nuanced. Indeed, presumed turn-over of microbial communities within the GIT of black bears might serve as an evolutionary advantage, not in association with specialized digestion, but regarding adiposity. In humans and mice, dysbiosis (i.e., imbalance of native microbes within a community, often brought about through dietary shifts) and low GMB diversity can lead to increased capacity for energy harvest and obesity (Turnbaugh et al. 2006, Ley 2010, Tilg and Kaser 2011, Chatelier et al. 2013, Menni et al. 2017). Although obesity is linked to health ailments in humans (Cani et al. 2008, Scher et al. 2015), the need for black bears to undergo hyperphagia (i.e., physiological Stage IV) to rapidly gain weight is paramount for reproductive success and survival during torpor (i.e., physiological Stage I; Rogers 1976, Eiler et al. 1989). For example, previous research shows seasonal composition and structure of GMBs in brown bears differ between physiological stages, with gut microbiota promoting energy storage during hyperphagia (Sommer et al. 2016). Therefore, dynamic, low-diversity GMBs may provide an evolutionary advantage for wildlife species with unique life histories such as black bears.
With the increasing vulnerability of large carnivore populations to human-mediated environmental change worldwide, emphasis on bettering management and conserving these charismatic, ecologically significant species is paramount. Through the study of carnivore GMBs, opportunities exist to identify effects of anthropogenic pressures on carnivores not solely from a behavioral perspective or as a result of direct persecution, but also in revealing how carnivores respond physiologically to human pressures (e.g., habitat degradation, access to human foods [processed foods]) and potential consequences of those physiological responses to the health of carnivores. Moreover, by better understanding the GMBs of generalist hosts and how the GMB respond to environmental change, managers can consider the value of incorporating strategies to promote holobiont conservation (e.g., host and GMB diversity) when striving to create effective management plans for species coping with human-mediated environmental change. As complexities of wildlife GMB co-evolution continue to be discovered, ecologists and conservationists alike must consider a more holistic approach to wildlife conservation in which wildlife and their GMBs are managed as a multigenomic organism (i.e., gut microbiome translocation for species re-introduction, gut microbiome diversity as a marker for host health, see Carthey et al. 2020. Specifically, as I did not find clear distinctions between alpha and beta diversity results, colon/fecal samples could prove adequate representation of the overall GIT microbial consortia for some wildlife hosts.

1.4.2 Differentially enriched bacterial taxa in simplistic GIT

Results showed the colon had high levels of differently enriched taxa from unknown, restrained lineages of bacteria (Table 1.7). Only two of the top significantly enriched taxa
of the colon GMB were identified to the genus level, Escherichia/Shigella and Turicibacter. Similarly, although fewer enriched taxa were identified in the jejunum GMB community, every enriched taxa was from a distinct order; however, none were identified below the taxonomic rank of order (Table 1.7). Nonsignificant differences for alpha diversity between the jejunum and colon, yet differentially enriched taxa in these two GIT sites could be due to the limited niche space available due to the simplicity of the carnivore GIT. However, differential taxonomic enrichment in the jejunum and colon is most likely due to site-specific environmental differences between the jejunum and colon.

1.4.3 Ursid fecal/colon GMB display similarity across studied species

Although the number of bacterial taxa identified in the black bear GIT communities were lower compared to other Ursid species, I found the colon GMB of American black bears were similarly dominated by the phyla Firmicutes and Proteobacteria (Schwab and Gänzle 2011, Zhu et al. 2011, Sommer et al. 2016, Borbón-García et al. 2017, Song et al. 2017). Three differently enriched taxa of the colon GMB community, Turicibacter, Clostridium sensu stricto 1, and Escherichia/Shigella, were found previously to be major genera of captive Asiatic black bear and giant panda GMBs (Xue et al. 2015, Song et al. 2017).

Perhaps the most interesting discovery was the presence of the minor genus Ursidibacter from the family Pasteurellaceae, first sequenced from oral-cavity swabs collected from polar bear and brown bear (Dietz et al. 2015), which was present in several jejunum and colon samples in my study. Pasteurellaceae are often pathogenetic or at times commensal bacteria typically unable to survive in external environments and
are found in the upper respiratory tract, throat, reproductive tracts, and in the GIT of host vertebrate hosts (Christensen and Bisgaard 2008). Members of the family Pasteurellaceae almost all appear to be closely coupled to a single vertebrate host and are believed to be adapted to specific habitat (Christensen and Bisgaard 2008). Although to my knowledge, *Ursidibacter* has not been reported in other Ursid GMB communities since it was first sequenced in 2015, this could be due to the reference databases/versions used. Like the genus *Prevotella* in non-human primates (Ma et al. 2014, Yasuda et al. 2015), *Ursidibacter* may be a co-evolved bacteria of the family Ursidae and merits further investigation to better understand the potential co-evolutionary history of Ursidae and their GMB.

1.5 Conclusion

Although the present project was limited to characterizing the GMB community composition within two GIT sites, I hope this work will serve as a catalyst for future carnivore-GMB research. To further strengthen our understanding of wildlife-GMB co-evolution and the roles GMBs can play in wildlife conservation, I encourage researchers to continue to use harvested animals while also implementing multi-omic approach (i.e., metabolomic, transcriptomic) to not only determine GMB composition but also to link membership to the GMB’s functional roles, and the implications of different GMB communities for host fitness.
2. CHAPTER TWO: HUMAN-PROVISIONED FOODS REDUCE GUT MICROBIOME DIVERSITY IN WILD AMERICAN BLACK BEAR (Ursus americanus)

2.1 INTRODUCTION

2.1.1 Background

Despite the increased risk of transmission of infectious diseases with baiting (Sorensen et al. 2014), many wildlife agencies across the United States allow baiting with human-provisioned foods to facilitate the harvest of a diverse array of wildlife (e.g., white-tail deer [Odocoileus virginianus], elk [Cervus canadensis], American black bear [Ursus americanus], red fox [Vulpes vulpes fulvus]). Yet, the negative consequences of baiting to wildlife health may go beyond disease transmission. For instance, diet is one of the most influential determinants of a host’s gut microbiome (GMB), the mutualistic microbial communities located in the gastrointestinal tract (GIT) of a host (Muegge et al. 2011, Bokulich et al. 2016, Greene et al. 2018, McKenney et al. 2018b). In fact, mammalian GMBs play pivotal roles in host health including weight modulation, metabolic function, digestion, and immune system maintenance (Hooper et al. 2002, 2012, Nicholson et al. 2012, Menni et al. 2017). Additionally, the GMB can rapidly respond to dietary shifts by changing gene expression and metabolic pathways, increasing a host’s capacity to adapt to dietary changes (David et al. 2014). By contrast, the modern “Western diet” (often called the Standard American Diet, or SAD), high in processed carbohydrates, trans/saturated fats, artificial sweeteners, and high fructose-corn syrup, has led to a considerable depletion in microbial diversity and an increase in chronic diseases in humans (see Deehan and Walter 2016). The Western diet is associated with bowel
inflammation, arthritis, diabetes, and obesity (Manzel et al. 2013, Deehan and Walter 2016, Zinöcker and Lindseth 2018); and while the spatial and temporal extent of baiting may differ by state, together this diverse array of intentionally human-provisioned foods represents a Westernization of wildlife diets. For example, Michigan is one of 12 states that permits baiting as a tool in the harvest of American black bear, with unrestricted quantities of human-provisioned foods including dog and cat foods, corn products, and bakery/confectionery products such as jams, jellies, sweeteners, candies, and other cooked or commercially processed foods. Further, Michigan hunters/guides are permitted to begin baiting bears (three bait stations/hunter) 31 days prior to the season opening and throughout the season, meaning that human-provisioned foods are available on the landscape for ~78 days. Such practices expose wildlife to a variety of “unnatural” foods for extended periods of time, and could therefore cause shifts in GMB community composition that may alter micro-ecosystem functions and affect host health (Hooper et al. 2002, Turnbaugh et al. 2006).

American black bears present an exciting opportunity to explore the response of the GMB to dietary shifts resulting from the introduction of human-provisioned foods to wildlife diet. Though physiologically a carnivore (e.g., simple GIT), black bears tend to be omnivorous with plant matter comprising the majority of their diet (Beeman and Pelton 1980, Enders and Vander Wall 2012); and they undergo extreme seasonal dietary shifts due to a unique life history (Nelson et al. 1983, Stevens and Hume 1995). During the fall months when hunting and baiting occurs in Michigan, black bears enter a physiological phase of increased caloric intake and weight gain known as hyperphagia (Brody and Pelton 1988). However, although consumption of human-provisioned foods
can ensure high caloric intake, the physiological consequences of shifts in GMB diversity as a result of consuming a “Western diet” during hyperphagia is unknown. Here we address this knowledge gap by engaging hunters in citizen science through the collection of biological samples during the annual harvest of thousands of black bears in Michigan. The engagement of hunters as citizen scientists also allows for sampling regions of the GIT that are otherwise inaccessible through non-invasive sampling or traditional animal handling procedures, such as sampling the jejunum, a section of the small intestine harboring GMB communities that have not been previously analyzed in a wild carnivore. Collecting jejunum content provides a unique opportunity to compare the GMB of two functionally distinct regions of the GIT: the GMB of the jejunum, where the absorption of proteins, fats, and carbohydrates occurs (Borgström et al. 1957, Thomson et al. 2003); and the colon, where fiber fermentation typically occurs in hindgut fermenters (McNeil 1984). Given the functional differences between the two sites, sampling both regions allows for a longitudinal analysis of changes in microbiota communities across the GIT in response to human-provisioned foods.

2.1.2 Objectives

In the present study, I used high-throughput amplicon sequencing, stable isotope analysis, and citizen science to investigate the response of GMB communities in wild American black bears to human-provisioned foods in the Upper Peninsula of Michigan. I aimed to 1) estimate the proportional contributions of human-provisioned foods to the diets of black bears and 2) investigate the influence of human-provisioned foods on GMB community composition in two GIT regions (i.e., jejunum and colon) of harvested black bears. Based on previous research on model species investigating the influence of
Western foods on GMB diversity, I hypothesized that higher proportions of human-provisioned foods in black bear diets would significantly reduce GMB alpha and beta diversity of both GIT regions, and that each site would harbor unique microbial communities.

2.2 METHODS

2.2.1 Study area

This study occurred in the Upper Peninsula of Michigan (42,896 km²), which is bordered by Wisconsin to the West, Lake Superior to the North, and Lake Huron and Lake Michigan to the East and South (Figure 2.1). Land cover in the Upper Peninsula is dominated by conifer-hardwood forests, and the climate supports limited agricultural production. Human population density in the Upper Peninsula is relatively low (7.3 people/km²), representing roughly 3% of Michigan’s total human population. By contrast, the Upper Peninsula is home to an estimated 8,700-11,000 American black bears, accounting for nearly 80% of the total Michigan black bear population (Michigan Department of Natural Resources 2015).

2.2.2 Black bear hunting and sampling

I received an exemption from review by the Northern Michigan University Institutional Animal Care and Use Committee because my samples were collected from dead bears that were legally harvested by hunters that were not involved with my study. I collected samples with permission from individual hunters/guides under a State of Michigan, Department of Natural Resources – Wildlife Division – Scientific Collector's Permit (Permit #SC 1613).
Biological samples were collected from legally harvested black bears during the 2018 black bear hunting season (September 10-October 26). Bear hunting in the Upper Peninsula was under a zone and quota system for six Bear Management Units (i.e., Amasa, Baraga, Bergland, Carney, Gwinn, Newberry), and samples were collected from each unit. Throughout the 2018 black bear harvest season in Michigan, hunters harvested 1,141 black bears in the Upper Peninsula, during which time 86 ± 1% of hunters primarily used bait (Frawley 2018). Of the hunters that used bait, 70% relied on baked goods, corn products, and grains, including all hunters and guides that contributed samples to the current project (Table 2.1).

Hunters/guides collected jejunum and colon contents for microbiome analysis, as well as guard hair samples for stable isotope analysis to estimate diet for the harvested bears and one roadkill bear (n=35; collected within 30 minutes of death; for complete methods see Appendix E: Supplemental 1). Briefly, samples were collected from the colon with sterile tongue depressors, and jejunum samples were collected by making an incision 16 inches below the phyloric sphincter and pouring intestine contents into sterile 15mL centrifuge tubes containing 7mL of 95% ethanol. All samples were stored at room temperature until microbial DNA were extracted (~50 days). Black bear sex and the types of baits each hunter/guide used to attract bears were recorded for each bear (Table 2.1). Sex was recorded and Michigan Department of Natural Resources provided age data from teeth they collected per their harvest registration protocols (Table 2.1). Bears were later classified into three age-classes based on previously published literature: yearlings (=1 year old), subadults (2–3 years old), and adults (≥ 4 years old; Lee and Vaughan 2005).
2.2.3 Stable isotope analysis

I removed hair follicles from guard hair samples, as lipids present therein can cause bias in stable isotope values of $\delta^{13}C$ (DeNiro and Epstein 1977). As it takes time for dietary changes to be assimilated into a consumer’s tissue and subsequently detected in hair samples (Oelze 2016), and bear guard hairs grow at a rate of a 1.5 cm/month (Felicetti et al. 2004), hair samples were cut into three equal segments, with the segment closest to the root used to estimate assimilated diet from July to harvest. Guard hair samples were sent to Cornell University Stable Isotope Laboratory for stable carbon and nitrogen isotope analysis following standard methods using a Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer. Stable isotope values are expressed in delta ($\delta$) notation, as a ratio relative to PeeDee Belemnite limestone (C) and atmospheric nitrogen (N) as parts per mil (‰), such that:

$$\delta X = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1,000$$

2.2.4 Diet estimations

To determine the proportional contribution of human-provisioned foods (i.e., bait and corn) to black bear diets, I a priori identified four isotopically distinct and possibly important black bear food categories: natural vegetation, terrestrial animal matter, bait and corn. Although corn is sometimes used as bear bait and is available across the landscape via deer feeders, I differentiated bait (e.g., baked goods, breakfast cereals, confectionaries) from corn because these food categories have distinct isotopic signatures (Table 2.2). For all sources, I estimated the proportional contribution of each food category to the diet of black bears by comparing carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope values derived from black bear hair samples, with stable isotope values of the
four major dietary food categories derived from the primary literature, which were based on a study in Northern Wisconsin, bordering the Upper Peninsula (Table 2.2; Kirby et al. 2017). I estimated composition of black bear diets using the Bayesian mixing model MixSIAR (version 3.1.10; Stock and Semmens 2013).

Prior to estimating diet, I confirmed suitable isotopic mixing space based on a visual assessment of an isotopic biplot, using raw isotope values for each black bear as well as the mean and standard deviation (±SD) of isotopic values of sources, which were corrected for discrimination (Figure 2.2). All models were fitted with Markov Chain Monte Carlo (MCMC) methods with uninformative priors to estimate posterior distributions for each food category (Carlin and Chib 1995). I used Gibbs sampling and applied 100,000 chain lengths, a burn-in of 50,000, and thinning of 50 across three chains. I created a candidate set of seven models to examine how isotopic variation was structured throughout the population (Table 2.3). The seven models included sex and age-class as categorical fixed effects incorporating process error × residual error, individuals as a random effect incorporating process error only (Stock and Semmens 2016), and the NULL model with no covariates with process error × residual error (Table 2.3). I used deviance information criterion (DIC) and leave-one-out cross-validation (LOO) to evaluate which model was most supported by the data, with the most conservative model having the lowest DIC value (Spiegelhalter et al. 2002) and the difference in LOO information criterion = 0 (dLOOic; Burnham and Anderson 2002, Stock et al. 2018). I confirmed model convergence by visually inspecting trace plots and with the Gelman-Rubin diagnostic (\( \hat{R} < 1.05 \) indicating convergence; Gelman and Rubin 1992). I reported
median posterior proportional contributions of each food category and 95% credible intervals (CI) for these estimates for the best ranked model.

2.2.5 Microbiome analysis

I used DNEasy PowerSoil Kits (QIAGEN, Hilden, Germany) to extract microbial DNA following a modified version of the manufactures protocol previously implemented by McKenney et al. 2017. I assessed DNA quantity/quality by spectrophotometric measurements using a Nanodrop-2000. I stored DNA extractions at -20°C and shipped on dry ice to Argonne National Laboratory for amplicon library preparation and multiplexed sequencing of bacterial 16S rRNA v4 gene region using the forward primer 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and the reverse primer 806R (5’-GGACTACHVGGGTWTCTAAT-3’).

I used the bioinformatics platform QIIME2 (Quantitative Insights Into Microbial Ecology; version 2019.4; Bolyen et al. 2019) to join raw sequences and demultiplex. Using the DADA2 QIIME2 plugin (Callahan et al. 2016), sequences were denoised, filtered to remove chimeras and residual Phix reads, dereplicated, and amplicon sequence variants (ASVs) were called. I used a trained Naïve Bayes sklearn classifier (Bokulich et al. 2018) to classify organisms at the genus level with the SILVA 99 database for V4 region (version 132; Quast et al. 2012), and then aligned sequences with MAFFT (Katoh and Standley 2013). I removed sequences identified as chloroplast, mitochondrial, and Archaea, as well as sequences unidentified below kingdom level, and any unassigned sequences (see Appendix G: Supplemental 3 for QIIME2 pipeline).
2.2.6 Statistical analysis

All statistical analyses and visualizations were conducted using R (version 3.6.2; R. Core Team 2018) and Rstudio (version 1.2.5033; Allaire 2012) with packages qiime2R (version 0.99.12; Bisanz 2018), phyloseq (version 1.28.0; McMurdie and Holmes 2013), microbiome (version 1.6.0; Lahti and Shetty 2012), picante (R, version 1.8; Kembel et al. 2010), lme4 (version 1.1-2; Bates et al. 2019), MASS (version 7.3-51.4; Ripley et al. 2019), and vegan (version 2.5-5; Oksanen et al. 2019) as outlined in Appendix I: Supplemental 5 for R script.

After rarefication and removal of samples from two bears with unknown age-classes, I retained 61 samples for analysis. From the rarified sequencing data, I calculated Shannon index (richness and evenness; Hill 1973), observed ASVs (OASVs; richness), and Faith’s Phylogenetic Diversity (PD; phylogeny; Faith 1992). To test the responses of the alpha diversity metrics to human-provisioned foods, I used generalized linear mixed models (GLMMs) for OASV counts with Negative Binomial distribution to account for overdispersion, and linear mixed models (LMMs) with a Gaussian distribution for Shannon diversity and log transformed PD. Distributions were determined via the fitdistr function in the package fitdistrplus (version 1.0-14; Delignette-Muller and Dutang 2015) and QQ plots.

I selected the models that best explained variation in GMB diversity based on the Akaike’s information criterion corrected for small samples (AICc). For each alpha diversity metric, I applied a model with all variables (main effects only), simple models with interaction (between two variables), and the null models (alpha ~ 1 + [1|Subject]; Table 2.4). I considered models with ΔAICc ≤ 2 as possibilities to explain the variation in
the data for the alpha diversity metrics with the R package *AICcmodavg* (version 2.2-2). Additionally, I calculated the conditional \( R^2_c \) and marginal \( R^2_m \) coefficients of determination for the top ranked models with the *performance* package (version 0.4.2; Lüdecke et al. 2019). The \( R^2_c \) represents the variance explained by both fixed and random factors (Nakagawa and Schielzeth 2013), while the \( R^2_m \) indicates how much of the model variance is explained by the fixed effects. GLMMs were performed using the *glmer* function for *OASVs* counts and *lmer* function for Shannon diversity and PD with the *lme4* package. I applied ANOVA with the *car* package to test for significant differences alpha diversity and obtained the \( \chi^2 \) and \( p \)-values for each top ranked model.

The proportional contribution of human-provisioned foods to the diet of black bears was investigated in two ways: a “human foods” category was created by adding together the proportional contributions of bait and corn to the diet of each bear; and the proportional contributions of bait and corn were examined separately (Table 2.4). Individuals were considered random effects, and the interactions between human-provisioned foods×GIT, bait×GIT, or corn×GIT or age-class were investigated to determine if one site or age-class was more affected than the others, with regards to GMB diversity relative to the consumption of human-provisioned foods. All main effects were also considered in models that included interactions. For all models, I checked residuals to confirm model requirements (e.g., normality, heteroscedasticity, residuals).

To determine if significant differences existed in GMBs among black bears with different proportional contributions of human-provisioned foods to the diet, I calculated the nonparametric quantitative/qualitative phylogenetic weighted and unweighted Unifrac distance matrices from rarified data, and fit weighted Unifrac vectors on principle.
coordination analysis (PCoA) plots. Unifrac distances are beta diversity measures that include phylogenetic information to identify factors explaining differences among microbial communities (Lozupone and Knight 2005, Lozupone et al. 2011). I performed permutational multivariate analysis of variance (perMANOVA; Anderson 2001), followed by multivariate homogeneity analysis (PERMDISP; Anderson 2006), to examine the overall effect of human-provisioned foods on GMB composition. I considered differences to be statistically significant at $p=0.05$ for all analyses.

### 2.3 RESULTS

#### 2.3.1 Proportion of human foods in black bear diet

Stable isotope values ranged from $-19.31\%$ to $-26.45\%$ for $\delta^{13}C$ and from $2.29\%$ to $5.97\%$ for $\delta^{15}N$ (Figure 2.2; Table 2.5). All mixing models converged to the posterior distribution, although model 7 (with the main effect of age-class, and the random effect of individual-level variation and process error) had the strongest support (Table 2.3). Model 7 had the lowest dLOOic and received 87.5% of the Akaike weight. Model 7 also showed considerable among-individual variation in the median proportional contribution of total human-provisioned foods (bait and corn) to the diet of bears, which ranged from $3.5\%$ to $56.1\%$ and from $2\%$ to $29.6\%$ for bait and corn, respectively (Figure 2.3; Table 2.6). Subadults consumed the greatest amounts of bait and corn, with each contributing a median of $35.2\%$ (range 4.4-57.3%) and $5.2\%$ (1-12.7%), respectively. Vegetation was the dominant food source for all age-classes, contributing a median estimated proportional contribution of $58.9\%$ (range 42-74%) to yearlings, and $47.1\%$ (range 34.1-58.7%) to sub-adults and $56.3\%$ (range 42.9-67.1%) to adult black bear (Figure 2.3; Table 2.6). Terrestrial meats contributed the least to bear diets overall.
2.3.2 The influence of human foods on GMB diversity

The best supported (G)LMM model for all alpha diversity indices included only the proportional contribution of corn (Table 2.4). Shannon diversity did not differ with different proportional contributions of corn, and corn had little explanatory value ($\chi^2 = 0.36, p = 0.55, R^2_c=0.02, R^2_m=0.006$). However, I found that PD and OASVs significantly decreased as the proportional contributions of corn to the diet of black bears increased (PD: $\chi^2 = 5.72, p = 0.02, R^2_c=0.14, R^2_m=0.09$; OASVs: $\chi^2 = 5.63, p = 0.02$, $R^2_c=0.25, R^2_m=0.11$; Figure 2.4). Specifically, for every percentage increase in corn in the diet, the back-transformed PD decreased by 89.13 and OASVs decreased by 3.17.

When considering the proportional contributions of bait, corn and GIT site to GMB diversity, I found significant differences in beta diversity, as measured by weighted and unweighted Unifrac distances (Table 2.7). However, PERMDISP results indicated a significant difference in homogeneity of dispersion between proportional contributions of corn for weighted Unifrac distances ($F=2.29, p=0.004$) and significant difference in homogeneity of dispersion between proportional contributions of corn (F=8.54, $p=0.002$), bait (F=4.37, $p=0.018$), and GIT site (F=12.77, $p=0.004$) for unweighted Unifrac distances. The significance of GIT site, and proportional contributions of corn and bait on GMB composition may be an artifact of within-group dispersion, leaving the perMANOVA results for unweighted Unifrac difficult to interpret. Additionally, ordination plots show little clustering of either weighted or unweighted Unifrac distances, regardless of location or differences in the proportional contributions of human-provisioned foods to the diet of black bears (Figure 2.5).
2.4 DISCUSSION

2.4.1 Diet of black bears comprised of high quantities of human-provisioned foods

The diets of black bears in the Upper Peninsula of Michigan were dominated by natural vegetation, although human-provisioned foods were an important food source for many individuals, ranging from 6.1% to 62.3% of the diet during the fall (Figure 3). The average proportional contribution of human-provisioned foods to the diet of black bears in the Upper Peninsula of Michigan was 37%, similar to historic populations of highly food-conditioned bears in Yosemite National Park (35%; Hopkins et al. 2014) and heavily baited black bear populations in Wisconsin (40%; Kirby et al. 2017).

2.4.2 Human-provisioned foods negatively affect GMB diversity

My results indicate that black bears consuming higher proportions of human-provisioned foods experience substantial reduction in microbial diversity as indicated by the PD and OASVs values for jejunum and colon GMBs. Black bears with diets containing less corn displayed higher levels of both microbial taxonomic richness and subsequently phylogenetic microbial diversity, while diets high in corn were correlated with reduced diversity in the GMBs. No other predictors of GMB community composition (e.g. age-class, GIT site) were found to influence GMB diversity. Beta diversity results were more ambiguous due to significant difference in homogeneity of dispersion among the considered covariates (e.g., bait, corn, GIT) and a lack of clear clustering in ordination plots (Figure 2.5).

Many of the baits used by the hunters and guides who contributed samples to this study were food items composed of domestic corn products such as high-fructose corn syrup, including artificial sweeteners and processed carbohydrates – all foods linked to
shifts in the GMB of humans (Suez et al. 2014, Singh et al. 2017; Table 2.1). Corn uses the $\delta^{13}$C enriched C$_4$ photosynthetic pathway with $\delta^{13}$C values ranging from -9‰ to -19‰, whereas C$_3$ plants (natural vegetation) show more depleted $\delta^{13}$C values ranging from -22‰ to -35‰ (Figure 2.2; Koch 2007). Although not statistically analyzed in this study, black bear diets containing higher proportions of corn were enriched with $\delta^{13}$C and appeared to align with lower PD and OAVSs (Figure 2.6), indicating that human-provisioned foods with higher amounts of corn derivatives negatively influence GMB communities.

GMB diversity could have several health-related implications, as the composition of the GMB can affect the efficacy of nutrition uptake from food (Bäckhed et al. 2005). Moreover, while some GMB functions can be carried out by several bacterial taxa, other functions involve a unique interaction with specific bacteria (Faith et al. 2014). For example, researchers have linked the reduction of GMB diversity and dysbiosis to alterations in GMB metabolic function (Turnbaugh et al. 2009) and liver disease (Chatelier et al. 2013, Schnabl and Brenner 2014). Previous studies have documented negative health affects linked to reduced GMB diversity in humans and other non-human primates, suggesting that a GMB comprising diverse taxa and functions is required to maximize the symbiotic relationship between wildlife hosts and their GMBs and to prevent dysbiosis requires a multifaceted and diverse GMB (Petersen and Round 2014).

2.4.3 The GMB of the jejunum and colon were not significantly different

The physiology of black bears could explain why neither the jejunum nor colon GMBs were differentially effected by the proportional contribution of human-provisioned foods to the diet of black bears. For example, species that consume a similar diet as black bears
but have more complex gastrointestinal tracts such as domestic pigs (*Sus scrofa domesticus*) and aye-ayes (*Daubentonia madagascariensis*) exhibit significant longitudinal differences in GIT microbial diversity (Xiao et al. 2018, Greene and McKenney 2018). By contrast, black bears’ simplistic, short GIT lacks a cecum or appendix, which prolong gut transit time and thus serve as a microbial reservoir. The lack of distinction in GMBs between black bear GIT sites may therefore result from the dominance of similar microbial taxa (i.e., opportunists that are resilient to disturbance).

### 2.4.4 Implications for wildlife management

Michigan Department of Natural Resources and other wildlife agencies consider hunting to be an important source of funding and opportunity to promote outdoor recreation, as well as a tool for helping to maintain target populations sizes and for reducing human-wildlife conflict. As wildlife managers are tasked with maintaining healthy, harvestable wildlife, I encourage wildlife managers to consider the potential negative health implications of baiting wildlife with foods that epitomize the “Western diet”, and how baiting policies may affect wildlife GMBs and subsequent host fitness. For instance, while black bears are the intended consumer of bait across the Upper Peninsula landscape, other species are known to visit and consume bear bait, including wolves (*Canis lupus*), marten (*Martes americana*), and fisher (*Martes pennant*) (personal communication with hunters). Thus, the effects of human-provisioned foods on wildlife GMBs could be widespread across ecosystems, beyond the impacts on black bears discussed here. Although current policies limit some types of foods permitted for bear baiting (i.e., chocolates are prohibited), stricter regulation of food types and bait quantity could reduce the loss of microbial diversity and potential function.


Michigan Department of Natural Resources. 2015. Memorandum to the Natural Resources Commission: bear regulations and license quotas Wildlife Conservation Order Amendment No. 2 of 2015. Michigan Department of Natural Resources, Lansing, Michigan, USA.


Accessible online at: http://conserver.iugo-cafe.org/user/brice.semmens/MixSIAR.


Trevelline, B. K., S. S. Fontaine, B. K. Hartup, and K. Kohl. 2019. Conservation biology needs a microbial renaissance: A call for the consideration of host-associated


Table 1.1 | Sex, age/age-class, and gastrointestinal site collection for each black bear (*Ursus americus*) sampled during the 2018 the Upper Peninsula of Michigan black bear hunting season.

<table>
<thead>
<tr>
<th>Bear</th>
<th>GIT</th>
<th>Sex</th>
<th>Age</th>
<th>Age Class</th>
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</thead>
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<td>Yearling</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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Table 1.2 | Wald $\chi^2$ tests for Alpha diversity model selection.

A. Faith’s phylogenetic diversity

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<th>BIC</th>
<th>logLik</th>
<th>deviance</th>
<th>$\chi^2$</th>
<th>Chi Df</th>
<th>Pr(&gt;Chisq)</th>
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B. Shannon diversity

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A. Simpson diversity

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<td>168.74</td>
<td>-67.92</td>
<td>135.85</td>
<td>0.35</td>
<td>1</td>
<td>0.56</td>
</tr>
<tr>
<td>model3</td>
<td>9</td>
<td>153.14</td>
<td>172.14</td>
<td>-67.57</td>
<td>135.14</td>
<td>0.71</td>
<td>1</td>
<td>0.40</td>
</tr>
<tr>
<td>model4</td>
<td>10</td>
<td>154.94</td>
<td>176.05</td>
<td>-67.47</td>
<td>134.94</td>
<td>0.20</td>
<td>1</td>
<td>0.65</td>
</tr>
</tbody>
</table>
### Table 1.3 | Wald's $\chi^2$ results for $\alpha$-diversity on top LMM model performed on alpha diversity indices.

<table>
<thead>
<tr>
<th>Effect</th>
<th>$\chi^2$</th>
<th>df</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIT</td>
<td>2.18</td>
<td>1.00</td>
<td>0.14</td>
</tr>
<tr>
<td>Sex</td>
<td>0.09</td>
<td>1.00</td>
<td>0.76</td>
</tr>
<tr>
<td>Age Class</td>
<td>7.17</td>
<td>2.00</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

**A. Faith's Phylogenetic diversity $R^2_c = 0.214, R^2_m = 0.141**

**B. Shannon diversity $R^2_c = 0.041, R^2_m = 0.025**

<table>
<thead>
<tr>
<th>Effect</th>
<th>$\chi^2$</th>
<th>df</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIT</td>
<td>0.13</td>
<td>1.00</td>
<td>0.72</td>
</tr>
<tr>
<td>Sex</td>
<td>0.06</td>
<td>1.00</td>
<td>0.80</td>
</tr>
<tr>
<td>Age Class</td>
<td>1.36</td>
<td>2.00</td>
<td>0.51</td>
</tr>
</tbody>
</table>

**C. Simpson diversity $R^2_c = 0.025, R^2_m = 0.017**

<table>
<thead>
<tr>
<th>Effect</th>
<th>$\chi^2$</th>
<th>df</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIT</td>
<td>0.0002</td>
<td>1.00</td>
<td>0.99</td>
</tr>
<tr>
<td>Sex</td>
<td>0.118</td>
<td>1.00</td>
<td>0.67</td>
</tr>
<tr>
<td>Age Class</td>
<td>.84</td>
<td>2.00</td>
<td>0.66</td>
</tr>
</tbody>
</table>

### Table 1.4 | emmeans for LMM models of Faith's phylogenetic diversity comparing black bear age-classes.

Age Class P-value adjustment: Tukey method for comparing a of three estimates

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Estimate</th>
<th>SE</th>
<th>df</th>
<th>t ratio</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yearling-Subadult</td>
<td>-0.30</td>
<td>0.17</td>
<td>27.7</td>
<td>-1.73</td>
<td>0.21</td>
</tr>
<tr>
<td>Yearling-Adult</td>
<td>0.07</td>
<td>0.18</td>
<td>28</td>
<td>0.39</td>
<td>0.92</td>
</tr>
<tr>
<td>Subadult-Adult</td>
<td>0.37</td>
<td>0.15</td>
<td>28.1</td>
<td>2.52</td>
<td>0.05*</td>
</tr>
</tbody>
</table>

Degrees-of-freedom: Satterthwaite
Confidence level: 0.95

### Table 1.5 | perMANOVA results for Bray-Curtis dissimilarity between GIT sites, sexes, and age-class.

<table>
<thead>
<tr>
<th>Factor</th>
<th>meanSqs</th>
<th>df</th>
<th>$R^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. GIT site</td>
<td>1.00</td>
<td>1</td>
<td>0.04</td>
<td>0.003**</td>
</tr>
<tr>
<td>B. Sex</td>
<td>0.49</td>
<td>1</td>
<td>0.02</td>
<td>0.51</td>
</tr>
<tr>
<td>C. Age-class</td>
<td>0.50</td>
<td>3</td>
<td>0.04</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

### Table 1.6 | perMANOVA results for weighted Unifrac distance between GIT sites, sexes, and age-class

<table>
<thead>
<tr>
<th>Factor</th>
<th>meanSqs</th>
<th>df</th>
<th>$R^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. GIT site</td>
<td>0.14</td>
<td>1</td>
<td>0.07</td>
<td>0.03*</td>
</tr>
<tr>
<td>B. Sex</td>
<td>0.01</td>
<td>1</td>
<td>0.005</td>
<td>0.007*</td>
</tr>
<tr>
<td>C. Age-class</td>
<td>0.03</td>
<td>3</td>
<td>0.03</td>
<td>0.04*</td>
</tr>
</tbody>
</table>
Table 1.7 | Microbial taxa significantly ($p<0.05$) enriched in black bear (*Ursus americanus*) jejunum (top) versus colon (bottom), as determined by LEfSe analysis.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>genus</th>
<th>log (LDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Jejunum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Bacilli</td>
<td>Bacillales</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>4.68</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Pseudomonadales</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>4.07</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Alphaproteobacteria</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>4.05</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Actinobacteria</td>
<td>Actinobacteria</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>3.9</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Actinobacteria</td>
<td>Actinobacteria</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>3.85</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Betaproteobacteria</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>3.84</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Alphaproteobacteria</td>
<td>Rhizobiales</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>3.76</td>
</tr>
<tr>
<td><strong>Colon</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Enterobacteriales</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>4.86</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Enterobacteriales</td>
<td>Enterobacteriaceae</td>
<td>Unidentified</td>
<td>4.86</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Enterobacteriales</td>
<td>Enterobacteriaceae</td>
<td>Escherichia_Shigella</td>
<td>4.84</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Alphaproteobacteria</td>
<td>Rhizobiales</td>
<td>Xanthobacteriaceae</td>
<td>Unidentified</td>
<td>4.75</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Erysipelotrichia</td>
<td>Erysipelotrichales</td>
<td>Erysipelotrichaceae</td>
<td>Turicibacter</td>
<td>4.74</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>4.74</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Erysipelotrichia</td>
<td>Erysipelotrichales</td>
<td>Erysipelotrichaceae</td>
<td>Unidentified</td>
<td>4.73</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Erysipelotrichia</td>
<td>Unidentified</td>
<td>Erysipelotrichaceae</td>
<td>Unidentified</td>
<td>4.72</td>
</tr>
<tr>
<td>Epsilonbacteraeota</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>4.46</td>
</tr>
<tr>
<td>Epsilonbacteraeota</td>
<td>Campylobacteria</td>
<td>Campylobacteriales</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>4.46</td>
</tr>
<tr>
<td>Epsilonbacteraeota</td>
<td>Campylobacteria</td>
<td>Campylobacteriales</td>
<td>Helicobacteriaceae</td>
<td>Helicobacter</td>
<td>4.45</td>
</tr>
<tr>
<td>Epsilonbacteraeota</td>
<td>Campylobacteria</td>
<td>Campylobacteriales</td>
<td>Helicobacteriaceae</td>
<td>Unidentified</td>
<td>4.45</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridia</td>
<td>Clostridiales</td>
<td>Clostridiaceae1</td>
<td>Clostridiumsensustricto</td>
<td>4.43</td>
</tr>
<tr>
<td>Epsilonbacteraeota</td>
<td>Campylobacteria</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>Unidentified</td>
<td>4.43</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridia</td>
<td>Clostridiales</td>
<td>Peptostreptococcaceae</td>
<td>Unidentified1</td>
<td>4.17</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridia</td>
<td>Clostridiales</td>
<td>Peptostreptococcaceae</td>
<td>Unidentified2</td>
<td>3.65</td>
</tr>
</tbody>
</table>
**APPENDIX B**

**CHAPTER TWO TABLES**

*Table 2.1* | Sex, harvest method, and types of baits used for black bears (*Ursus americanus*) harvested in the Upper Peninsula of Michigan 2018.

<table>
<thead>
<tr>
<th>Bear</th>
<th>Sex</th>
<th>Age Class</th>
<th>Method</th>
<th>Bait</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Male</td>
<td>Yearling</td>
<td>Bait</td>
<td>Cookie dough, dog food</td>
</tr>
<tr>
<td>B2</td>
<td>Female</td>
<td>Yearling</td>
<td>Hounds</td>
<td>Cherries, granola, corn, dog food</td>
</tr>
<tr>
<td>B3</td>
<td>Male</td>
<td>Yearling</td>
<td>Bait</td>
<td>Fruit loops, grease</td>
</tr>
<tr>
<td>B4</td>
<td>Male</td>
<td>Yearling</td>
<td>Bait</td>
<td>Granola, nuts, raisins, cherries, skittles, marshmallows, dog food</td>
</tr>
<tr>
<td>B5</td>
<td>Male</td>
<td>Yearling</td>
<td>Bait</td>
<td>Granola, nuts, raisins, cherries, skittles, marshmallows, dog food</td>
</tr>
<tr>
<td>B6</td>
<td>Male</td>
<td>Yearling</td>
<td>Bait</td>
<td>Cheese, cookies, yoghurt, dog food</td>
</tr>
<tr>
<td>B7</td>
<td>Male</td>
<td>Yearling</td>
<td>Bait</td>
<td>Apples, dog food, blueberry pie filling, Bavarian cream, corn</td>
</tr>
<tr>
<td>B8</td>
<td>Male</td>
<td>Subadult</td>
<td>Road kill</td>
<td>-</td>
</tr>
<tr>
<td>B9</td>
<td>Female</td>
<td>Subadult</td>
<td>Bait</td>
<td>Apples, dog food, custard, blueberry pie, corn</td>
</tr>
<tr>
<td>B10</td>
<td>Male</td>
<td>Subadult</td>
<td>Bait</td>
<td>Cookie dough, dog food</td>
</tr>
<tr>
<td>B11</td>
<td>Female</td>
<td>Subadult</td>
<td>Bait</td>
<td>Cookie dough, dog food</td>
</tr>
<tr>
<td>B12</td>
<td>Male</td>
<td>Subadult</td>
<td>Bait</td>
<td>Breakfast cereal, dog food</td>
</tr>
<tr>
<td>B13</td>
<td>Female</td>
<td>Subadult</td>
<td>Bait</td>
<td>Circus peanuts, candy, lucky charms, sugar, gummies, dogfood</td>
</tr>
<tr>
<td>B14</td>
<td>Female</td>
<td>Subadult</td>
<td>Hounds</td>
<td>Granola, bread, popcorn, marshmallow, cherries</td>
</tr>
<tr>
<td>B15</td>
<td>Male</td>
<td>Subadult</td>
<td>Bait</td>
<td>Granola, nuts, raisins, cherries, skittles, marshmallows, dog food</td>
</tr>
<tr>
<td>B16</td>
<td>Male</td>
<td>Subadult</td>
<td>Bait</td>
<td>Granola, nuts, raisins, cherries, skittles, marshmallows, dog food</td>
</tr>
<tr>
<td>B17</td>
<td>Female</td>
<td>Subadult</td>
<td>Bait</td>
<td>Breakfast cereal, dog food</td>
</tr>
<tr>
<td>B18</td>
<td>Male</td>
<td>Subadult</td>
<td>Bait</td>
<td>Breakfast cereal, dog food</td>
</tr>
<tr>
<td>B19</td>
<td>Male</td>
<td>Subadult</td>
<td>Bait</td>
<td>Cheese, cookies, yoghurt, dog food</td>
</tr>
<tr>
<td>B20</td>
<td>Male</td>
<td>Subadult</td>
<td>Bait</td>
<td>Cheese, cookies, yoghurt, dog food</td>
</tr>
<tr>
<td>B21</td>
<td>Male</td>
<td>Subadult</td>
<td>Bait</td>
<td>Granola, cherries, honey, breakfast cereal</td>
</tr>
<tr>
<td>B22</td>
<td>Female</td>
<td>Subadult</td>
<td>Bait</td>
<td>Granola, cherries, honey, breakfast cereal</td>
</tr>
<tr>
<td>B23</td>
<td>Female</td>
<td>Adult</td>
<td>Hounds</td>
<td>Cherries, granola, corn, marshmallow</td>
</tr>
<tr>
<td>B24</td>
<td>Female</td>
<td>Adult</td>
<td>Hounds</td>
<td>Cherries, granola, corn, marshmallows</td>
</tr>
<tr>
<td>B25</td>
<td>Male</td>
<td>Adult</td>
<td>Hounds</td>
<td>Cherries, granola, corn, marshmallow</td>
</tr>
<tr>
<td>B26</td>
<td>Male</td>
<td>Adult</td>
<td>Hounds</td>
<td>Marshmallows, granola, bread, popcorn</td>
</tr>
<tr>
<td>B27</td>
<td>Female</td>
<td>Adult</td>
<td>Bait</td>
<td>Cheese, cookies, yoghurt, dog food</td>
</tr>
<tr>
<td>B28</td>
<td>Male</td>
<td>Adult</td>
<td>Bait</td>
<td>Circus peanuts, canes, lucky charms, powdered sugar, gummies</td>
</tr>
<tr>
<td>B29</td>
<td>Female</td>
<td>Adult</td>
<td>Bait</td>
<td>Circus peanuts, canes, lucky charms, powdered sugar, gummy fish</td>
</tr>
<tr>
<td>B30</td>
<td>Male</td>
<td>Adult</td>
<td>Bait</td>
<td>Cherries, bread, frosting, relish, apples, hard candies</td>
</tr>
<tr>
<td>B31</td>
<td>Male</td>
<td>Adult</td>
<td>Bait</td>
<td>Granola, cherries, honey, breakfast cereal</td>
</tr>
<tr>
<td>B32</td>
<td>Male</td>
<td>Adult</td>
<td>Bait</td>
<td>Granola, cherries, honey, breakfast cereal</td>
</tr>
<tr>
<td>B33</td>
<td>Female</td>
<td>Adult</td>
<td>Bait</td>
<td>Apples, dog food, blueberry pie filling, Bavarian cream, corn</td>
</tr>
<tr>
<td>B34</td>
<td>Female</td>
<td>Unknown</td>
<td>Bait</td>
<td>Cookie dough, dog food</td>
</tr>
<tr>
<td>B35</td>
<td>Female</td>
<td>Unknown</td>
<td>Hounds</td>
<td>Cherries, granola, corn, dog food</td>
</tr>
</tbody>
</table>
Table 2.2 | Mean raw isotopic values ($\delta^{13}$C and $\delta^{15}$N) ± standard deviation (SD) and trophic fractionation factors ($\Delta\delta^{13}$C and $\Delta\delta^{15}$N) ± standard deviation (SD) for diet sources used to determine the proportional contributions of the four major dietary sources (Kirby et al. 2017).

<table>
<thead>
<tr>
<th>Source</th>
<th>n</th>
<th>$\delta^{13}$C</th>
<th>$\delta^{15}$N</th>
<th>$\Delta\delta^{13}$C, $\Delta\delta^{15}$N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Vegetation</td>
<td>122</td>
<td>29.28 (1.90)</td>
<td>1.49 (0.13)</td>
<td>3.4 (0.2) 2.4 (0.2)</td>
</tr>
<tr>
<td>Terrestrial meat</td>
<td>34</td>
<td>26.66 (1.13)</td>
<td>2.06 (0.80)</td>
<td>2.1 (0.1) 3.9 (0.3)</td>
</tr>
<tr>
<td>Bait</td>
<td>27</td>
<td>25.61 (1.60)</td>
<td>3.94 (0.65)</td>
<td>4.1 (0.3) 2.8 (0.2)</td>
</tr>
<tr>
<td>Corn</td>
<td>24</td>
<td>12.07 (0.08)</td>
<td>6.98 (0.98)</td>
<td>3.4 (0.2) 2.4 (0.2)</td>
</tr>
</tbody>
</table>

Table 2.3 | Summary of stable isotope mixing models explaining variation in black bear ($Ursus americanus$) diets.

<table>
<thead>
<tr>
<th>Model</th>
<th>Covariate</th>
<th>Effect</th>
<th>Error</th>
<th>DIC</th>
<th>dLOOic</th>
<th>SE (dLOOic)</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Ageclass, Individual</td>
<td>Fixed, Random</td>
<td>process</td>
<td>0.642</td>
<td>0</td>
<td>NA</td>
<td>0.875</td>
</tr>
<tr>
<td>2</td>
<td>Individual</td>
<td>Random</td>
<td>process</td>
<td>14.25</td>
<td>5</td>
<td>3.9</td>
<td>0.072</td>
</tr>
<tr>
<td>6</td>
<td>Sex, Individual</td>
<td>Fixed, Random</td>
<td>process</td>
<td>14.61</td>
<td>5.6</td>
<td>3.7</td>
<td>0.053</td>
</tr>
<tr>
<td>1</td>
<td>NULL</td>
<td>NULL</td>
<td>process×residual</td>
<td>36.68</td>
<td>47.3</td>
<td>8.6</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Sex</td>
<td>Fixed</td>
<td>process×residual</td>
<td>40.33</td>
<td>48.2</td>
<td>8.5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Age-class</td>
<td>Fixed</td>
<td>process×residual</td>
<td>39.1</td>
<td>51.4</td>
<td>9.4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Sex, Age-class</td>
<td>Fixed, Fixed</td>
<td>process×residual</td>
<td>43.3</td>
<td>52.5</td>
<td>9.1</td>
<td>0</td>
</tr>
</tbody>
</table>

Model 7 had the lowest dLOOic and received 87.5% of the Akaike weight, indicating a 87.5% probability it was the best model.
Faith's Phylogenetic Diversity

<table>
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Shannon Diversity

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Table 2.5 | Raw isotope values (‰) derived from black bears (*Ursus americanus*) guard hairs used to estimate the proportional contributions of human foods to diet.

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Table 2.6 | Posterior estimated mean, standard deviation, and quantiles of the proportional contributions of four food items categories to the diet of black bear (*Ursus americanus*) for age-class and each individual bear harvested in the Upper Peninsula of Michigan (%).

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<td>0.299</td>
<td>0.494</td>
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<td>B28</td>
<td>Bait</td>
<td>0.16</td>
<td>0.097</td>
<td>0.022</td>
<td>0.144</td>
<td>0.382</td>
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<tr>
<td></td>
<td>Corn</td>
<td>0.128</td>
<td>0.052</td>
<td>0.032</td>
<td>0.126</td>
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<tr>
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<td>0.072</td>
<td>0.005</td>
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<td>Vegetation</td>
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<td>0.066</td>
<td>0.479</td>
<td>0.638</td>
<td>0.736</td>
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<tr>
<td>B29</td>
<td>Bait</td>
<td>0.156</td>
<td>0.092</td>
<td>0.023</td>
<td>0.144</td>
<td>0.361</td>
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<tr>
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<td>0.106</td>
<td>0.046</td>
<td>0.024</td>
<td>0.103</td>
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<tr>
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<td>Terrestrial</td>
<td>0.084</td>
<td>0.075</td>
<td>0.005</td>
<td>0.062</td>
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<tr>
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<td>Vegetation</td>
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<td>0.064</td>
<td>0.508</td>
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<td>B30</td>
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<td>Corn</td>
<td>0.205</td>
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<td>0.056</td>
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<tr>
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<td>Terrestrial</td>
<td>0.097</td>
<td>0.082</td>
<td>0.007</td>
<td>0.074</td>
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<tr>
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<td>Vegetation</td>
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<td>0.083</td>
<td>0.297</td>
<td>0.484</td>
<td>0.616</td>
</tr>
<tr>
<td>B31</td>
<td>Bait</td>
<td>0.309</td>
<td>0.176</td>
<td>0.03</td>
<td>0.3</td>
<td>0.659</td>
</tr>
<tr>
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<td>Corn</td>
<td>0.167</td>
<td>0.07</td>
<td>0.034</td>
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<tr>
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<td>Terrestrial</td>
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<td>0.031</td>
<td>0.238</td>
<td>0.566</td>
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<tr>
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<td>0.06</td>
<td>0.026</td>
<td>0.131</td>
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<td>0.12</td>
<td>0.006</td>
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<td>0.024</td>
<td>0.168</td>
<td>0.406</td>
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<tr>
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<td>0.108</td>
<td>0.048</td>
<td>0.024</td>
<td>0.106</td>
<td>0.206</td>
</tr>
<tr>
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<td>0.087</td>
<td>0.005</td>
<td>0.071</td>
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<tr>
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<td>0.068</td>
<td>0.47</td>
<td>0.619</td>
<td>0.731</td>
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</table>
Table 2.7 | perMANOVA results for weighted and unweighted Unifrac distances between GIT sites, sexes, and proportional contributions of corn and bait (** <0.01) (*<0.05).

<table>
<thead>
<tr>
<th>Weighted</th>
<th>Factor</th>
<th>meanSqs</th>
<th>df</th>
<th>R²</th>
<th>p</th>
</tr>
</thead>
<tbody>
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<td>A. GIT site</td>
<td>0.14</td>
<td>1</td>
<td>0.08</td>
<td>0.04*</td>
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</tr>
<tr>
<td>B. Corn</td>
<td>0.12</td>
<td>1</td>
<td>0.06</td>
<td>0.04*</td>
<td></td>
</tr>
<tr>
<td>C. Bait</td>
<td>0.1</td>
<td>1</td>
<td>0.05</td>
<td>0.02*</td>
<td></td>
</tr>
<tr>
<td>D. Corn×GIT</td>
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<td>1</td>
<td>0.005</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Unweighted</th>
<th>Factor</th>
<th>meanSqs</th>
<th>df</th>
<th>R²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. GIT site</td>
<td>0.31</td>
<td>1</td>
<td>0.06</td>
<td>0.001**</td>
<td></td>
</tr>
<tr>
<td>B. Corn</td>
<td>0.31</td>
<td>1</td>
<td>0.03</td>
<td>0.001**</td>
<td></td>
</tr>
<tr>
<td>C. Bait</td>
<td>0.2</td>
<td>1</td>
<td>0.02</td>
<td>0.003*</td>
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</tr>
<tr>
<td>D. Corn×GIT</td>
<td>0.1</td>
<td>1</td>
<td>0.008</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.1 | Mean relative abundance of the major taxa found within the jejunum and colon. "Minor" indicates the combined taxa with <1% relative abundance of each site. (A) The major phylum present within the jejunum and colon and (B) major genus. Mean relative abundance of the major taxa found within three age-classes of (C) the major phylum and (D) major genus.
Figure 1.2 | GIT site and age-class have distinct microbiota communities. NMDS plots from (A) Bray-Curtis dissimilarity showing more defined clustering of colon (blue) and a larger amount of dissimilarity between jejunum (green), however, age-class does not appear to have distinct pattern. perMANOVA results for Bray-Curtis showed significant difference between GIT sites ($R^2=0.04$, $p=0.003$) and age-class ($R^2=0.04$, $p=0.002$), however, PERMDISP results indicated significant difference in homogeneity for GIT ($F=12.37$, $p=0.002$) and age-class ($F=5.26$, $p=0.011$). PCoA plots of (B) weighted Unifrac distances of jejunum (green) and colon (blue). perMANOVA results for weighted Unifrac showed significant difference between GIT sites ($R^2=0.07$, $p=0.03$), sex ($R^2=0.005$, $p=0.007$), and age-class ($R^2=0.04$, $p=0.001$).
Figure 1.3 | Phylogenetic heat trees representing the community structure as observed within (A) jejunum and (B) colon samples from rarified dataset. Taxonomic hierarchy is of ASVs classification up to the genus level. Node width is proportional to the number of ASVs classified as that taxon and edge size is proportional to the number of reads. Color represents the number of ASVs assigned to each taxon (abundance). Within the (A) jejunum, we see a higher level of phylogenetic diversity whereas the (B) colon displays a smaller number of phyla. Black stars are bacteria indicated to be differently significant within each site via LEfSe. Although the colon has over double the amount of enriched taxa, the jejunum appears to have a higher level of phylogenetic variability for enriched taxa.
Figure 1.4 | Box plots with standard deviations for Faith's phylogenetic diversity for each age-class. Faith's phylogenetic diversity was significantly different between subadults and adults.
Figure 2.1| LANDFIRE vegetation cover type map of the Upper Peninsula of Michigan. American black bears (*Ursus americanus*) were harvested throughout the Upper Peninsula during the 2018 Michigan bear hunting season (September 10-October 26).
Figure 2.2 | Distribution of black bear (*Ursus americanus*) stable isotope values (•) in δ13C ‰ and δ15N ‰ isotopic space relative to mean ± SD for four food sources with trophic discrimination factors applied. Guard hairs were collected from American black bear located in the Upper Peninsula of Michigan in 2018.
Figure 2.3 | Boxplots of median (lines in boxes = median, box boundaries = 50 % credible intervals, error bars = 95 % CI) proportional contributions of each food source to black bear diet in the Upper Peninsula of Michigan calculated with MixSIAR for three age-classes. (A) boxplots with all four food categories foods and (B) human-provisioned foods combined (bait & corn).
Figure 2.4 | Relationship between the proportional contribution of corn and (A) Faith's PD, and (B) observed ASVs in two gastrointestinal sites. For every percentage increase in corn in the diet, PD decreased by 89.13 units and OASVs decreased by 3.17 units.
Figure 2.5 | (A) PCoA Ordination plots of GMB composition of black bear GIT with varying proportional contributions of (A) corn and (B) bait for weighted Unifrac and (C) corn and (D) bait for unweighted Unifrac distance matrices. Triangles indicate jejunum GMB and circles indicate colon GMB.
Figure 2.6 | Relationship between the proportional contribution of $\delta^{13}C$ to (A) Faith’s PD and (B) observed ASVs in two gastrointestinal sites.
**APPENDIX E**

**SUPPLEMENTAL 1: SAMPLE COLLECTION PROTOCOL**

*Please collect samples immediately after bear has been killed.*

**PHASE 1: FECAL COLLECTION**

1. Remove GREEN baggie labelled "FECAL" containing GREEN gloves.
2. With GREEN gloves on, remove popsicle stick from wrapping. Do not let the popsicle stick touch anything other than your gloved hand at this time.
3. Insert the popsicle stick into bear anus, placing fecal sample into the GREEN lid vial. Repeat until vial is half full (replace cap on vial in-between filling to minimize outside exposure). Secure cap tightly when sampling is complete.
4. Place vial back into small bag labelled "FECAL" and place small bag into main baggie.
5. Dispose of used popsicle stick and GREEN gloves. Please ensure all of phase one is complete and the black gloves and used popsicle sticks are disposed of before starting phase 2.

**PHASE 2: HAIR COLLECTION**

1. Before skinning bear, remove a small clump of hair (10+ hairs) from between the bear’s shoulders.
2. Place envelope into the Ziploc bag. Now the bear is ready to start being field-dressed! The last sample can be collected after the stomach and intestines are removed.

**PHASE 3: JEJUNUM COLLECTION**

1. After guts have been removed, isolate the stomach and small intestine (see back for full diagram). NOTE: the intestines are held in a bundle with thin connective tissue. You may need to trim a bit of this connective tissue to sample the jejunum.
2. Stretch the provided string from bottom of stomach (end labelled “stomach”), down the length of the intestines until string is tight (end labelled “jejunum”). Remove small baggie labelled “JEJUNUM” with sticker and BLUE gloves.
3. With PURPLE gloves on, make an incision in the intestine at the “jejunum” end of the string.
4. Pour sample out of the incision from the intestines side (the side connected to the rest of the intestines NOT the side closest to stomach) into the BLUE lid vial until the vial is half full (replace cap on vial in-between filling to minimize outside exposure). Secure cap tightly when sampling is complete.
5. Place vial back into small bag labelled “JEJUNUM”. Place small bag into main baggie.
6. Dispose of used BLUE gloves. Fill out label on main Ziploc bag.
7. With sharpie provided, please fill out datasheet located on front of large Ziploc bag. Be sure to fill out DNR seal Number so we can age bear.

**IMPORTANT**

Cross-contamination between the two vial samples can alter the results. To avoid this, we have color-coordinated the materials needed for each step. Please do not reuse any of the materials between phases!
APPENDIX F

SUPPLEMENTAL 2: DNA EXTRACTION PROTOCOL

Prior to starting:
- Check for precipitate in Solution C1. [If precipitate has formed, head at 60°C until precipitate dissolves]
- Pre-fill microtubes:
  - Tube1: 60μL Solution C1
  - Tube2: 250μL Solution C2
  - Tube 3: 200μL Solution C3
  - Tube4: Shake Solution C4 to mix; 1200μL Solution C4 (set 1,000μL micropipette to 600μL and administer twice)
- Do not fill Tube 5 or Tube 6 prior to use
- Get ice bath ready for 2-8°C incubation
- Set heat block to 65°C.
- Need clean spatulas, razor blades, weigh boats / weigh paper (use 95% ethanol for tools). Clean surfaces with bleach solution.

Things to remember:
- Keep everything sterile!
- Please label tubes with S##F for fecal samples and S##J for jejunum samples
- Place * on final microtube
- When weighing out sample, please go below the ethanol line.
- Tip: you might want to pre-cut 1000μL tips for extracting jejunum fluid
- A ★ by the steps indicates you are moving solutions into next Tube

Label (per run):

<table>
<thead>
<tr>
<th>Tube1</th>
<th>Tube2</th>
<th>Tube3</th>
<th>Tube4</th>
<th>Tube5</th>
<th>Tube6</th>
</tr>
</thead>
<tbody>
<tr>
<td>beads</td>
<td>+C1</td>
<td>+C2</td>
<td>+C3</td>
<td>+C4</td>
<td>column+C6</td>
</tr>
</tbody>
</table>

Modified DNA extraction from stool using QIAGEN DNEasy PowerSoil Kit

1. Add 60 μL Solution C1 to PowerBead Tube. (Do this when you are preparing the other microtubes).
2. Add 0.25 g of stool to PowerBead Tube. Vortex ~3s to mix. (Please record weight).
3. Heat at 65°C for 10 minutes.
5. Vortex at maximum speed for 10 min.
6. Centrifuge at 10,000x g for 30 s (to pellet stool particles).
7. Pipette supernatant from Tube1 into Tube2 (this should be pre-filled with 250μL of C2 solution). (400-500 μL supernatant.)
8. Vortex for 1 min.
9. Incubate at 2-8°C for 5 minutes.
10. Centrifuge at 10,000x g for 1 min.
11. Avoiding the pellet, pipette up to 600 uL supernatant into Tube3 (this should be pre-filled with 200 μL of Solution C3).
12. Vortex briefly.
13. Incubate at 2-8°C for 5 minutes.
14. Centrifuge at 10,000x g for 1 min.
15. Avoiding the pellet, pipette up to 750 μL supernatant into Tube4 (this should be pre-filled with 1200 μL of Solution 4).

16. Vortex for 5 s.

★ Tube5

17. Load 657 μL of supernatant C4 mixture from Tube4 into an Empty MB Spin Column (Tube5 with no additional solution) and centrifuge at 10,000x g for 1 min. Discard flow-through.

18. Repeat step 17 twice, OR until all of the sample has been processed.

19. Add 500 μL of Solution C5 into Tube5. Centrifuge at 10,000x g for 30 s.

20. Discard flow-through. Centrifuge again at 10,000x g, for 1 minute.

★ Tube6

21. Carefully place the MB Spin Column into a clean 2-mL Collection Tube (Tube6). Avoid splashing any Solution C5 onto the column.

22. Add 100 μL of Solution C6 directly to the center of the white filter membrane.

23. Close cap and incubate at Room Temp for 5 minutes.

24. Centrifuge at 10,000x g for 30 s.

25. Elute a second time (pipette solution at bottom of Tube6 directly onto white filter membrane again and centrifuge again don’t need to incubate again though).

26. SAVE FILTRATE (Tube6 with solution at bottom) AND DISCARD COLUMN. PLEASE UNTIL ALL SAMPLES ARE DONE FOR THE DAY TO DO NANODROP

27. Samples is now ready to have DNA measured and recorded in Dr. Lindsay’s lab via Nanodrop.

28. Store DNA product at -20°C.
### QIIME2 version: 2019.4
### Samples are from Argonne National Laboratory###
# Pipeline adapted from qiime2 tutorial "Atacama soil microbiome" & "Moving Pictures"
# samples are EMP-Paired end multiplexed sequences with new primer set
# w/ barcodes read forward & no longer reversed in demux step

################################ Import data into QIIME2 ################################

## import sequences
qiime tools import
--type EMPPairedEndSequences
--input path Reads
--output-path paired-end-sequences.qza #you can name this whatever you want
##output artifact: paired-end-sequences.qza

############################### Demultiplexing Sequences ################################
#you will need metadata/mapping file #
qiime demux emp-paired
--m-barcodes-file Meta.tsv
--m-barcodes-column BarcodeSequence
--p-no-golay-error-correction
--i-seqs paired-end-sequences.qza
--o-per-sample-sequences demuxseq.qza
--o-error-correction-details demux-detail.qza

# make a summary visualization
qiime demux summarize
--i-data demuxseq.qza
--o-visualization demuxseq.qzv

##with the .qzv file we will go to qiime2view online and look at the quality of our reads

################## Denoising sequences with DADA2 plugin ##################
# prior to denoising, look at demux.qzv to determine if/where to trim sequences
# you will need to have r installed in your qiime2 environment
#if R is not installed be sure to be in the qiime2 environment
# denoising

qiime dada2 denoise-paired \
--i-demultiplexed-seqs demuxseq.qza \
--p-trim-left-f 0 \
--p-trim-left-r 0 \
--p-trunc-len-f 150 \
--p-trunc-len-r 150 \
--o-table table.qza \
--o-representative-sequences rep-seqs.qza \
--o-denoising-stats denoising-stats.qza

#output artifacts: table.qza, rep-seqs.qza, denoising-stats.qza
# you will now have artifacts containing the
# feature table and corresponding feature sequences.
# You can generate summaries of those as follows

# summary visualization table for determining sample depth for rarifying
qiime feature-table summarize \
--i-table table.qza \
--o-visualization table.qzv \
--m-sample-metadata-file Meta.tsv
#output visualization: table.qzv
#sampling depth:18257
## remember this is before we remove contaminate

# make visualization artifacts of rep seq
qiime feature-table tabulate-seqs \
--i-data rep-seqs.qza \
--o-visualization rep-seqs.qzv
#output visualization: rep-seq.qzv

# view denoising stats
qiime metadata tabulate \
--m-input-file denoising-stats.qza \
--o-visualization denoising-stats.qzv
#output visualization: denoising-stats.qzv

################ now we can start using the "moving pictures tutorial" starting at

################ Taxonomic Analysis sklearn ################

## Training classifier
# https://docs.qiime2.org/2019.4/data-resources/
# https://docs.qiime2.org/2019.4/tutorials/feature-classifier/
## we use the SILVA reference database 515/806
# https://www.arb-silva.de/download/archive/qiime
## download SILVA_132_ or whatever the newest version is.
## put SILVA folder in Projects

## import reference otus

```bash
qiime tools import
--type 'FeatureData[Sequence]'
--input-path SILVA_132_99_16S.fna
--output-path SILVA_OTU.qza
```

## Import reference taxonomy file

```bash
qiime tools import
--type 'FeatureData[Taxonomy]'
--input-format HeaderlessTSVTaxonomyFormat
--input-path taxonomy_7_levels.txt
--output-path ref-taxonomy.qza
```

## Extract reference reads

```bash
qiime feature-classifier extract-reads
--sequences SILVA_OTU.qza
--p-f-primer GTGCCAGCMGCGCGGTAA
--p-r-primer GGACTACHVGGGTWTCTAAT
--p-trunc-len 150
--p-min-length 100
--p-max-length 400
--o-reads ref-seqs.qza
```

## Train the classifier

```bash
qiime feature-classifier fit-classifier-naive-bayes
--i-reference-reads ref-seqs.qza
--i-reference-taxonomy ref-taxonomy.qza
--o-classifier classifier.qza
```

## Test Classifier

```bash
qiime feature-classifier classify-sklearn
--i-classifier classifier.qza
--i-reads rep-seqs.qza
--o-classification taxonomySILVA.qza
```

##### fixing white spaces

```bash
qiime tools export
--input-path taxonomySILVA.qza
--output-path taxonomy-with-spaces
```
qiime metadata tabulate \
--m-input-file taxonomy-with-spaces/taxonomy.tsv \
--o-visualization taxonomy-as-metadata.qzv

qiime tools export \
--input-path taxonomy-as-metadata.qzv \
--output-path taxonomy-as-metadata

qiime tools import \
--type 'FeatureData[Taxonomy]' \
--input-path taxonomy-as-metadata/metadata.tsv \
--output-path -taxonomy-without-spaces.qza

# create visualization
qiime metadata tabulate \
--m-input-file taxonomy-without-spaces.qza \
--o-visualization taxonomySILVA.qzv

## Filtering

# filter out mitochondria and chloroplast
qiime taxa filter-table \
--i-table table.qza \
--i-taxonomy taxonomy-without-spaces.qza \
--p-exclude mitochondria \
--o-filtered-table table-filter.qza

qiime taxa filter-table \
--i-table table-filter.qza \
--i-taxonomy taxonomy-without-spaces.qza \
--p-exclude chloroplast \
--o-filtered-table clean-table.qza

# get rid of unassigned
qiime taxa filter-table \
--i-table clean-table.qza \
--i-taxonomy taxonomy-without-spaces.qza \
--p-exclude Unassigned \
--o-filtered-table clean-table-unassigned-rm.qza

# remove Bacteria only assigned
qiime taxa filter-table \
--i-table clean-table-unassigned-rm.qza \
--i-taxonomy taxonomy-without-spaces.qza \
--p-mode exact \
--p-exclude D_0__Bacteria \

--o-filtered-table clean-table-unassigned_UNKNOWN-rm.qza

# S remove Arch only assigned
qiime taxa filter-table
--i-table clean-table-unassigned_UNKNOWN-rm.qza
--i-taxonomy taxonomy-without-spaces.qza
--p-exclude D_0_Archaea
--o-filtered-table clean-table-unassigned_UNKNOWN_Arch-rm.qza

# barplot
qiime taxa barplot
--i-table clean-table-unassigned_UNKNOWN_Arch-rm.qza
--i-taxonomy taxonomy-without-spaces.qza
--m-metadata-file Meta.tsv
--o-visualization taxa-bar-plotsSILVA-clean2.qzv

# determine depth
qiime feature-table summarize
--i-table clean--table-unassigned_UNKNOWN_Arch-rm.qza
--o-visualization clean--table-unassigned_UNKNOWN_Arch-rm.qzv
--m-sample-metadata-file Meta.tsv
##1050

########### Generating a tree for phylogenetic diversity analyses with clean data ###########

## Filter
qiime feature-table filter-seqs
--i-data rep-seqs.qza
--i-table clean-table-unassigned_UNKNOWN_Arch-rm.qza
--o-filtered-data filtered-rep-seq.qza

##root
qiime phylogeny align-to-tree-mafft-fasttree
--i-sequences filtered-rep-seq.qza
--o-alignment aligned-rep-seqs.qza
--o-masked-alignment masked-aligned-rep-seqs.qza
--o-tree -filterd-unrooted-tree.qza
--o-rooted-tree filtered-rooted-tree.qza
library(microbiome) ## data analysis
library(qiime2R) # import data
library(phyloseq) # also the basis of data object. Data analysis and visualization
library(vegan) # some utility tools
library(data.table) # alternative to data.frame
library(dplyr) # data handling
library(tidyverse)
library(ggpubr) ## plotting
library(ggplot2)
library(mctoolsr)
library(picante) ## faith's PD
library(see)
library(Rmisc)## graphing

setwd("~/Desktop/Projects/Bear/Bear-R/CLEAN/FINAL")

### Import & create phyloseq dataframe with qiime2R and QIIME2 artifacts ####

## Following Tutorial: Integrating QIIME2 and R for data visualization and analysis using qiime2R by J. Bisanz
## you will need
# 1.) Metafile.tsv (alpha_tableR.tsv) -the alpha_table file will need to have the second row removed and the # in front of SampleID removed for it to read okay
# 2.) taxonomy.qza
# 3.) table.qza
# 4.) rooted.qza

## import artifacts & metadata file
metadata<-read_tsv("Metafile.tsv")
SVs<-read_qza("table.qza")
taxonomy<-read_qza("taxonomy.qza")
taxtable<-taxonomy$data %>% as_tibble() %>% separate(Taxon, sep=";", c("Domain", "Phylum", "Class", "Order", "Family", "Genus", "Species")) # convert the table into a tabular split version
tree<--read_qza("rooted-tree.qza")

## Create the phyloseq object
phy_obj<--phyloseq(
  otu_table(SVs$data, taxa_are_rows = T),
  phy_tree(tree$data),
  tax_table(as.data.frame(taxtable) %>% select(-Confidence) %>%
    column_to_rownames("Feature.ID") %>% as.matrix()), #moving the taxonomy to the
  sample_data(metadata %>% as.data.frame() %>% column_to_rownames("SampleID")))

## view data table
datatable(tax_table(phy_obj))

##### Clean Taxonomy table #####
## Rename NAs to last known group
tax.clean<--data.frame(tax_table(phy_obj))
for (i in 1:7){ tax.clean[,i]<--as.character(tax.clean[,i])
tax.clean[is.na(tax.clean)]<--"
for (i in 1:nrow(tax.clean)){
  if (tax.clean[i,2] == ""){
    kingdom<--paste("Kingdom_", tax.clean[i,1], sep = "")
tax.clean[i, 2:7]<--kingdom
  } else if (tax.clean[i,3] == ""){
    phylum<--paste("Phylum_", tax.clean[i,2], sep = "")
tax.clean[i, 3:7]<--phylum
  } else if (tax.clean[i,4] == ""){
    class<--paste("Class_", tax.clean[i,3], sep = "")
tax.clean[i, 4:7]<--class
  } else if (tax.clean[i,5] == ""){
    order<--paste("Order_", tax.clean[i,4], sep = "")
tax.clean[i, 5:7]<--order
  } else if (tax.clean[i,6] == ""){
    family<--paste("Family_", tax.clean[i,5], sep = "")
tax.clean[i, 6:7]<--family
  } else if (tax.clean[i,7] == ""){
    tax.clean$Species[i]<--paste("Genus",tax.clean$Genus[i], sep = "_")
  }
}
## import new taxonomy table
tax_table(phy_obj)<--as.matrix(tax.clean)

## view
datatable(tax_table(phy_obj))
##### Rename uncultured

tax.clean2 <- data.frame(tax_table(phy_obj))

for (i in 1:7) { tax.clean2[,i] <- as.character(tax.clean2[,i])
for (i in 1:nrow(tax.clean2)) {
  if (tax.clean2[i,2] == "uncultured") {
    kingdom <- paste("Kingdom_", tax.clean2[i,1], sep = "")
    tax.clean2[i, 2:7] <- kingdom
  } else if (tax.clean2[i,3] == "uncultured") {
    phylum <- paste("Phylum_", tax.clean2[i,2], sep = "")
    tax.clean2[i, 3:7] <- phylum
  } else if (tax.clean2[i,4] == "uncultured") {
    class <- paste("Class_", tax.clean2[i,3], sep = "")
    tax.clean2[i, 4:7] <- class
  } else if (tax.clean2[i,5] == "uncultured") {
    order <- paste("Order_", tax.clean2[i,4], sep = "")
    tax.clean2[i, 5:7] <- order
  } else if (tax.clean2[i,6] == "uncultured") {
    family <- paste("Family_", tax.clean2[i,5], sep = "")
    tax.clean2[i, 6:7] <- family
  } else if (tax.clean2[i,7] == "") {
    tax.clean2$Species[i] <- paste("Genus", tax.clean2$Genus[i], sep = " ")
  }
}

## import new taxonomy table

tax_table(phy_obj) <- as.matrix(tax.clean2)

## view new table

datatable(tax_table(phy_obj))

## save phyloseq object

saveRDS(phy_obj, "~/Desktop/Projects/Bear/Bear-R/CLEAN/FINAL/physeq.rds")

## if you ever want to pull back in
phy_obj <- readRDS("physeq.rds")

##### Alpha Diversity #####

## Equal sample sums
set.seed(9242) ## ensures rarifies the same each time script is run

summary(sample_sums(phy_obj)) ## helps determine depth for rarifying

## rarefying: we already know our depth: 1050 so rarefy to that
phyb.rar <- rarefy_even_depth(phy_obj, sample.size = 1050)
## lost one sample: S100J
summary(sample_sums(phyb.rar)) ## checking to see they all have the same sequence depth
any(taxa_sums(phy_obj)== 0) # making sure we don't have any sequences not in at least one samples
### run this is you do have 0's:ps1a <- prune_taxa(taxa_sums(phyb.rar) > 0, phyb.rar)

## pull metadata from physeq object
sam.meta <- meta(phyb.rar)

## put variables in particular order
sam.meta$GIT <- factor(sam.meta$GIT, levels=c("Jejunum", "Colon"))
sam.meta$AgeClass <- factor(sam.meta$AgeClass, levels=c("Yearling", "Subadult", "Adult", "Unknown"))

## Add the rownames as a new column for easy integration later.
sam.meta$sam_name <- rownames(sam.meta)

#### Non-phylogenetic diversities: Shannon ####
## calculated with microbiome package
div_shan <- microbiome::alpha(phyb.rar, index = "shannon")

#### Non-phylogenetic diversities: Simpson ####
## calculated with microbiome package
div_sim <- microbiome::alpha(phyb.rar, index="diversity_inverse_simpson")

#### Phylogenetic diversity: Faith's PD ####
# Phylogenetic diversity is calculated using the picante package.

## pull ASV table
phyb.rar.asvtab <- as.data.frame(phyb.rar@otu_table)

## pull tree
phyb.rar.tree <- phyb.rar@phy_tree

## We first need to check if the tree is rooted or not
phyb.rar@phy_tree
rooted so we are good to go

## Getting the data ready

```
div_pd <- pd(t(phyb.rar.asvtab), phyb.rar.tree, include.root=T)
```

# `t(ou_table)` transposes the table for use in picante and the tree file comes from the first code we used to read tree file (see making a phyloseq object section)

## Add the rownames to diversity table

```
div_pd$sam_name <- rownames(div_pd)
```

## STEP 4p. merge all of the alphas into one file

```
merged_table<-merge(div_pd, div_shan, by = "sam_name", all=T)
merged_table2<-merge(merged_table, sam.meta, by = "sam_name", all=T)
alpha_table <- merge(merged_table2, div_sim, by = "sam_name", all=T)
```

```
datatable(alpha_table) ## this now has all alpha measures in one datatable!
```

produces summary tables for diversity indices for age-class 

**note:** only one yearling female will not analyze

## females

### Community composition

## filter

```
pseq.rel <- microbiome::transform(phyb.rar, "compositional")
```

## merge to phylum rank

```
phlyum <- tax_glom(pseq.rel, taxrank = "Phylum")
ntaxa(phlyum)
#23
```

## melt

```
phylum_melt<- psmelt(phlyum)
```

```
unique(phylum_melt$Phylum)
#23
```

## get summary statistics phyla GIT

```
p_abund<-summarySE(phylum_melt, measurevar = "Abundance", groupvars =c("Phylum", "GIT"))
```

## remove 0 abundance

```
p_abund$Abundance[p_abund$Abundance==0] <- NA
p_abund<-p_abund[complete.cases(p_abund$Abundance),]
p_abund<- p_abund %>%
```

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```r
mutate_if(is.numeric, round, digits = 5)
unique(p_abund$Phylum)

## ageclass

## remove unknown ages
phylum_melt2 <- phylum_melt
phylum_melt2$AgeClass[phylum_melt2$Ages == 0] <- NA
phylum_melt2 <- phylum_melt2[complete.cases(phylum_melt2$Age),]
age_p_abund <- summarySE(phylum_melt2, measurevar = "Abundance", groupvars = c("Phylum", "AgeClass"))
age_p_abund <- age_p_abund %>%
  mutate_if(is.numeric, round, digits = 5)

## remove 0 abundance
age_p_abund$Abundance[age_p_abund$Abundance == 0] <- NA
age_p_abund <- age_p_abund[complete.cases(age_p_abund$Abundance),]
unique(age_p_abund$Phylum)

## genus

## merge to phylum rank
genus <- tax_glom(pseq.rel, taxrank = "Genus")
taxa(genus)
#

## melt
genus_melt <- psmelt(genus)

## get summary statistics genus GIT
g_abund <- summarySE(genus_melt, measurevar = "Abundance", groupvars = c("Genus", "GIT"))

g_abund$Abundance[g_abund$Abundance == 0] <- NA
g_abund <- g_abund[complete.cases(g_abund$Abundance),]
g_abund <- g_abund %>%
  mutate_if(is.numeric, round, digits = 5)
unique(g_abund$Genus)

## ageclass
genus_melt2 <- genus_melt
genus_melt2$AgeClass[genus_melt2$Ages == 0] <- NA
genus_melt2 <- genus_melt2[complete.cases(genus_melt2$Age),]
age_g_abund <- summarySE(genus_melt2, measurevar = "Abundance", groupvars = c("Genus", "AgeClass"))

## remove 0 abundance
```
age_g_abund$Abundance[age_g_abund$Abundance==0] <- NA
age_g_abund<-age_g_abund[complete.cases(age_g_abund$Abundance),]
age_g_abund<- age_g_abund %>%
    mutate_if(is.numeric, round, digits = 5)
unique(age_g_abund$Genus)

### LMER Analysis for Alpha diversity ######

library(lmerTest)
library(lme4)
library(car)
library(emmeans)
library(sjmisc)
library(sjPlot)
library(tidyverse)

### bear samples without age-class (n=4) have been removed from the analysis
## rename
alpha_table0<-alpha_table
alpha_table0$GIT<-factor(alpha_table0$GIT, levels=c("Jejunum", "Colon"))
alpha_table0$AgeClass<-factor(alpha_table0$AgeClass, levels=c("Yearling", "Subadult", "Adult"))

## remove unknown ages
alpha_table0$AgeClass[alpha_table0$Ages==0] <- NA
alpha_table0<-alpha_table0[complete.cases(alpha_table0$Age),]

#### LMER PD ####

## histogram
ggplot(alpha_table0,aes(x=PD))+geom_histogram()

## skewed right so log transform
## create intial models with maximum likelihood
pd_lme1<-lmer(log(PD)~GIT+Sex+AgeClass+(1|Subject),data=alpha_table0, REML=F)
pd_lme2<-lmer(log(PD)~GIT*Sex+Sex+AgeClass+(1|Subject),data=alpha_table0, REML=F)
pd_lme3<-lmer(log(PD)~GIT*AgeClass+Sex+GIT+AgeClass+(1|Subject),
data=alpha_table0, REML=F)
pd_lme4<-lmer(log(PD)~GIT+Sex+AgeClass+GIT*AgeClass+GIT*Sex+(1|Subject),
data=alpha_table0, REML=F)
## compare to determine best model
anova(pd_lme1, pd_lme2, pd_lme3, pd_lme4)

## best model run with REML=T
pd_lme1<-lmer(log(PD)~GI+Sex+AgeClass+(1|Subject),data=alpha_table0, REML=T)
summary(pd_lme1)

## run final model to get
Anova(pd_lme1)
## sig difference between GIT site and for age-class

## get R^2
library(performance)
performance::r2(pd_lme1)

## Estimated Marginal Means
library(emmeans)

emmeans(pd_lme1, pairwise~AgeClass,lmer.df="satterthwaite", adjust="tukey")
## sig difference is between adults and subadults

## Assumption 1 - Linearity

## Graphically, plotting the model residuals (the difference between the observed value and the model-estimated value) vs the predictor
alpha_table1<-alpha_table0
alpha_table1$log<-log(alpha_table1$PD)
Plot.Model.F.Linearity<-plot(resid(pd_lme1),alpha_table1$log)

## Assumption 2 Homogeneity of Variance
# Regression models assume that variance of the residuals is equal across groups.

# extracts the residuals and places them in a new column in our original data table
alpha_table1$lme10<-residuals(pd_lme1)
alpha_table1$baslme10 <-abs(alpha_table1$lme10) # creates a new column with the absolute value of the residuals
alpha_table1$lme102 <- alpha_table1$baslme10^2 # squares the absolute values of the residuals to provide the more robust estimate
pd_leven <- lm(lme102 ~ Subject, data=alpha_table1) # ANOVA of the squared residuals
anova(pd_leven) # displays the results

## visually
plot(pd_lme1) # creates a fitted vs residual plot

## Assumption 3: The residuals of the model are normally distributed.
# QQ plots
library(lattice)

qqmath(pd_lme1, id=0.05)
## overall looks good!!

### LMER Shannon ###

## histogram
ggplot(alpha_table0, aes(x=diversity_shannon)) + geom_histogram()

## create initial models with maximum likelihood
shan_lme1 <- lmer(diversity_shannon~GIT+Sex+AgeClass+(1|Subject), data=alpha_table0, REML=F)
shan_lme2 <- lmer(diversity_shannon~GIT+GIT*Sex+Sex+AgeClass+(1|Subject), data=alpha_table0, REML=F)
shan_lme3 <- lmer(diversity_shannon~GIT*AgeClass+Sex+GIT+AgeClass+(1|Subject), data=alpha_table0, REML=F)
shan_lme4 <- lmer(diversity_shannon~GIT+Sex+AgeClass+GIT*AgeClass+GIT*Sex+(1|Subject), data=alpha_table0, REML=F)

## compare to determine best model
anova(shan_lme1, shan_lme2, shan_lme3, shan_lme4)

## best model run with REML=T
shan_lme1 <- lmer(diversity_shannon~GIT+Sex+AgeClass+(1|Subject), data=alpha_table0, REML=T)

## run final model to get
Anova(shan_lme1)
## no sig
## get R^2
performance::r2(shan_lme1)

### Assumption 1 - Linearity

## Graphically, plotting the model residuals (the difference between the observed value and the model-estimated value) vs the predictor
Plot.Model.F.Linearity <- plot(resid(shan_lme1), alpha_table0$diversity_shannon)

### Assumption 2 Homogeneity of Variance
# Regression models assume that variance of the residuals is equal across groups.
#extracts the residuals and places them in a new column in our original data table
alpha_table2<-alpha_table0
alpha_table2$lme10<-residuals(shan_lme1)
alpha_table2$baslme10 <- abs(alpha_table2$lme10) # creates a new column with the absolute value of the residuals
alpha_table2$lme10^2 <- alpha_table2$baslme10^2 # squares the absolute values of the residuals to provide the more robust estimate
shan_leven <- lm(lme102 ~ Subject, data=alpha_table2) # ANOVA of the squared residuals
anova(shan_leven) # displays the results

## visually
plot(shan_lme1) # creates a fitted vs residual plot

## Assumption 3: The residuals of the model are normally distributed.

# QQ plots
qqmath(shan_lme1, id=0.05)
## overall looks good!!

##### LMER Simpson ####

## histogram
ggplot(alpha_table0,aes(x=diversity_inverse_simpson))+geom_histogram()
ggplot(alpha_table0,aes(x=log(diversity_inverse_simpson)))+geom_histogram()
#### transform with log as it is skewed

## create initial models with maximum likelihood
sim_lme1<- lmer(log(diversity_inverse_simpson)~GIT+Sex+AgeClass+(1|Subject),data=alpha_table0, REML=F)
sim_lme2<- lmer(log(diversity_inverse_simpson)~GIT*Sex+Sex+AgeClass+(1|Subject),data=alpha_table0, REML=F)
sim_lme3<- lmer(log(diversity_inverse_simpson)~GIT*AgeClass+Sex+GIT+AgeClass+(1|Subject), data=alpha_table0, REML=F)
sim_lme4<- lmer(log(diversity_inverse_simpson)~GIT+Sex+AgeClass+GIT*AgeClass+GIT*Sex+(1|Subject), data=alpha_table0, REML=F)

## determine best fit model
anova(sim_lme1, sim_lme2, sim_lme3, sim_lme4)

## lme1 best run with REML=T
```r
sim_lme1 <- 
  lmer(log(diversity_inverse_simpson) ~ GIT + Sex + AgeClass + (1|Subject), 
data = alpha_table0, REML = T)
summary(sim_lme1)

## run final model to get
Anova(sim_lme1)
## no sig

## get R^2
performance::r2(sim_lme1)

## Assumption 1 - Linearity

## Graphically, plotting the model residuals (the difference
# between the observed value and the model-estimated value) vs
# the predictor
alpha_table3 <- alpha_table0
alpha_table3$log <- log(alpha_table3$diversity_inverse_simpson)
Plot.Model.F.Linearity <- plot(resid(sim_lme1), alpha_table3$log)

## Assumption 2 Homogeneity of Variance
# Regression models assume that variance of the residuals
# is equal across groups.
# extracts the residuals and places them in a new column in our original data table
alpha_table3$lme10 <- residuals(sim_lme1)
alpha_table3$baslme10 <- abs(alpha_table3$lme10) # creates a new column with the absolute value of the residuals
alpha_table3$lme102 <- alpha_table3$baslme10^2 # squares the absolute values of the residuals to provide the more robust estimate
sim_leven <- lm(lme102 ~ Subject, data = alpha_table3) # ANOVA of the squared residuals
anova(sim_leven) # displays the results

## visually
plot(sim_lme1) # creates a fitted vs residual plot

## Assumption 3: The residuals of the model are normally distributed.

# QQ plots
qqmath(sim_lme1, id = 0.05)
## some deviation but overall looks good!!
```
### Beta Diversity ###

## Bray
remove unknowns

```
new_obj = subset_samples(phyb.rar, AgeClass != "Unknown")
new_obj.rel = subset_samples(q.rel, AgeClass != "Unknown")
```

pull out ASV abundances from our relative abundance dataframe and use meta meta
data file already created

```
ASV <- as(otu_table(new_obj), "matrix")
if(taxa_are_rows(new_obj)) {ASV <- t(ASV)}
ASVdf = as.data.frame(ASV)
```

### permanova

```
beta_bra = - vegdist(ASVdf, index = "bray")
```

```
br_permanova <- adonis(beta_bra ~ GIT + Sex + AgeClass, strata = alpha_table1$Subject, data = alpha_table1)
```

```
br_permanova
```

### Checking the homogeneity condition

#GIT
```
permutest(betadisper(beta_bra, alpha_table1$GIT), strata = Subject)
```

### jaccard
```
beta_ja <- vegdist(ASVdf, index = "jaccard", binary = T)
```

```
ja_permanova <- adonis(beta_ja ~ GIT + Sex + AgeClass, strata = alpha_table1$Subject, data = alpha_table1)
```

```
ja_permanova
```

### Checking the homogeneity condition

#GIT
```
permutest(betadisper(beta_ja, alpha_table1$GIT), strata = alpha_table1$Subject)
```

#age
permutest(betadisper(beta JA, alpha_table1$AgeClass), strata=alpha_table1$Subject)
## not sig

#### UniFrac ####

## remove unknowns
new_obj.rel = subset_samples(pseq.rel, AgeClass != "Unknown")
## weighted
unifrac.dist <- UniFrac(new_obj.rel,
    weighted = TRUE,
    normalized = TRUE,
    parallel = FALSE,
    fast = TRUE)

WU_permANOVA <- adonis(unifrac.dist ~ GIT+Sex+AgeClass,
    strata=alpha_table1$Subject, data = alpha_table1)
WU_permANOVA
## GIT sig

##### Checking the homogeneity condition

#GIT
permutest(betadisper(unifrac.dist, alpha_table1$GIT), strata=Subject)
## not significant!
permutest(betadisper(unifrac.dist, alpha_table1$Sex), strata=Subject)
## not significant
permutest(betadisper(unifrac.dist, alpha_table1$AgeClass), strata=Subject)
## not significant

## unweighted
ununifrac.dist <- UniFrac(new_obj,
    weighted = FALSE,
    parallel = FALSE,
    fast = TRUE)

unWU_permANOVA <- adonis(ununifrac.dist ~ GIT+Sex+AgeClass,
    strata=alpha_table1$Subject, data = alpha_table1)
unWU_permANOVA
## GIT significant

#GIT
permutest(betadisper(ununifrac.dist, alpha_table1$GIT), strata=Subject)
## significance for GIT so permanova result may be potentially explained by that.

permutest(betadisper(ununifrac.dist, alpha_table1$AgeClass), strata=Subject)
## not significant

##### Community composition Visualization GIT ####

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## bar plot to show mean relative abundance of each community at phyla and genus level.

### phyla

```r
p_abund$Phylum <- as.character(p_abund$Phylum)
unique(p_abund$Phylum)

# simple way to rename phyla with < 1% abundance
p_abund$Phylum[p_abund$Abundance <= 0.01] <- "Other"
unique(p_abund$Phylum)

## put phyla in order so it plots most abundant on bottom
p_abund$Phylum <- factor(p_abund$Phylum, levels = c("Other", "Actinobacteria", "Epsilonbacteraeota", "Proteobacteria", "Firmicutes"))

## plot
spatial_plot <- ggplot(data = p_abund, aes(x = GIT, y = Abundance, fill = Phylum, width = 0.8)) +
  coord_flip()
p1 <- spatial_plot + geom_bar(aes(), stat = "identity", position = "stack", width = 1) +
  scale_fill_manual(values = c("black", "gray", "deeppink4", "cyan", "forestgreen")) +
  theme_bw() +
  theme(legend.position = "bottom", axis.title = element_text(size = 9, family = "Arial"),
        legend.key.size = unit(0.2, "cm"),
        legend.key.text = element_text(size = 5, family = "Arial"),
        legend.title.align = 0.5,
        legend.key.width = unit(0.2, "cm"),
        legend.spacing.x = unit(0.2, "cm"),
        legend.spacing.y = unit(0.2, "cm"),
        legend.title = element_text(size = 9, family = "Arial"),
        axis.text.x = element_blank(),
        axis.ticks.x = element_blank(),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank()) +
  guides(fill = guide_legend(nrow = 1, byrow = TRUE, color = guide_legend(nrow = 1), reverse = TRUE, title.position = "top")) +
  xlab("") + ylab("")
p1
```

### genus

```r
unique(g_abund$Genus)
g_abund$Genus <- as.character(g_abund$Genus)

# simple way to rename phyla with < 1% abundance
g_abund$Genus[g_abund$Abundance <= 0.01] <- "Other"
unique(g_abund$Genus)
write_csv(g_abund, "g_abund.csv")

### put in order you want
g_abund$Genus <- factor(g_abund$Genus, levels = c("Other", "Helicobacter", "Moraxella", "Weissella", "Other", "Actinobacteria", "Epsilonbacteraeota", "Proteobacteria", "Firmicutes"))
```
"Staphylococcus","Sporosarcina","Family_Enterobacteriaceae","Family_Pasteurellaceae",
"Escherichia-Shigella","Terrisporobacter",
"Paeniclostridium","Romboutsia","Lactococcus","Turicibacter",
"Enterococcus",
"Cellulosilyticum","Bacillus","Family_Peptostreptococcaceae",
"Lactobacillus","Streptococcus",
"Clostridium sensu stricto 1","Sarcina")

spatial_plot2 <- ggplot(data=g_abund, aes(x=GIT, y=Abundance, fill=Genus, width=.8)) +
  coord_flip()
p2<-spatial_plot2 + geom_bar(aes(),stat="identity", position="stack", width =1) +
  scale_fill_manual(values =
c("black","lightpink","turquoise4","cyan2","steelblue4","lightblue","lightslateblue","blue 1","cadetblue3","gold1","khaki1","yellow4","lightgoldenrod1","limegreen","olivedrab2","springgreen4","lightgreen","palegreen4","darkseagreen2","green","forestgreen","darkgreen")) +
  theme_bw() +
  theme(legend.position="bottom", axis.title=element_text(size=9, family="Arial"), legend.key.size = unit(0.2, "cm"),
   legend.text = element_text(size=5, family="Arial"),
   legend.key.width=unit(0.2,'cm'), legend.spacing.x = unit(2, 'cm'), legend.title.align=0.5,
   legend.spacing.y=unit(.2, 'cm'), legend.title = element_text(size=9, family="Arial"),
   axis.text =element_text(color="black", family="Arial"),
   panel.grid.major = element_blank(), panel.grid.minor = element_blank()) +
  guides(fill=guide_legend(nrow=3, byrow=TRUE, color=guide_legend(nrow=3),reverse = TRUE, title.position="top")) +
  xlab("") + ylab("Mean Relative Abundance %")
p2
## plot together & save
tiff('Community.tiff', units="in", width=7, height=4, res=300, compression = 'lzw')
see::plots(p1,p2, n_columns = 1, tags=c("A", "B"))
dev.off()

#### Community composition Visualization Age class ####
# put phyla in order fso it plots most abundant on bottom
age_p_abund$Phylum <- as.character(age_p_abund$Phylum)
# simple way to rename phyla with < 1% abundance
age_p_abund$Phylum[age_p_abund$Abundance <= 0.01] <- "Other"
unique(age_p_abund$Phylum)

age_p_abund$Phylum <- factor(age_p_abund$Phylum, levels = c("Other",
"Actinobacteria","Tenericutes","Epsilonbacteraeota","Proteobacteria","Firmicutes"))
## plot

```r
spatial_plot3 <- ggplot(data=age_p_abund, aes(x=AgeClass, y=Abundance, fill=Phylum, width=.8)) +
  coord_flip()
```

```r
p3 <- spatial_plot3 + geom_bar(aes(), stat="identity", position="stack", width =1) +
  scale_fill_manual(values = c("black","gray", "yellow","deppink4", "cyan", "forestgreen")) +
  theme_bw() +
  theme(legend.position="bottom",axis.title=element_text(size=9, family="Arial"),legend.key.size = unit(0.2, "cm"),
  legend.key.text = element_text(size=5, family="Arial"),legend.title.align=0.5,
  legend.spacing.x = unit(.2, 'cm'),
  legend.spacing.y = unit(.2, 'cm'),
  axis.text.x =element_blank(),axis.ticks.x =element_blank(),
  panel.grid.major = element_blank(), panel.grid.minor = element_blank()) +
  guides(fill=guide_legend(nrow=1, byrow=TRUE, color=guide_legend(nrow=1), reverse = TRUE, title.position = "top")) +
  xlab("") + ylab("")
```

## genus

```r
age_g_abund$Genus <- as.character(age_g_abund$Genus)
```

```r
#simple way to rename phyla with < 1% abundance
age_g_abund$Genus[age_g_abund$Abundance <= 0.01] <- "Other"
unique(age_g_abund$Genus)
```

```r
### put in order you want
age_g_abund$Genus <- factor(age_g_abund$Genus, levels =
c("Other","Helicobacter","Family_Neisseriaceae","Bibersteinia",
"Moraxella","Weissella",
"Staphylococcus","Sporosarcina","Family_Pasteurellaceae","Escherichia-
Shigella","Terrisporobacter",
"Mycoplasma","Leuconostoc","Paeniclostridium","Romboutsia"
,"Lactococcus","Turicibacter",
"Enterococcus",
"Cellulosilyticum","Bacillus","Family_Peptostreptococcaceae",
"Lactobacillus","Streptococcus",
"Clostridium sensu stricto 1","Sarcina")
```

```r
spatial_plot4 <- ggplot(data=age_g_abund, aes(x=AgeClass, y=Abundance, fill=Genus, width=.8)) +
  coord_flip()
```

```r
p4 <- spatial_plot4 + geom_bar(aes(), stat="identity", position="stack", width =1) +
  scale_fill_manual(values =
c("black","lightpink","purple1","mediumorchid","turquoise4","cyan2","steelblue4","lightblue","lightslateblue","blue1","cadetblue3","gold1","orange3","khaki1","yellow4","lightg
```

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oldenrod1","limegreen", "olivedrab2", "springgreen4","lightgreen","palegreen4", "darkseagreen2", "green", "forestgreen", "darkgreen")+
  theme_bw()+
  theme(legend.position="bottom",axis.title=element_text(size=9,
family="Arial"),legend.key.size = unit(0.2, "cm"),
  legend.text = element_text(size=5, family="Arial"),
  legend.key.width=unit(0.2,'cm'),legend.spacing.x = unit(.2,
'cm'),legend.title.align=0.5,
  legend.spacing.y=unit(2, 'cm'),legend.title = element_text(size=9, family="Arial"),
  axis.text =element_text(color="black", family="Arial"),
  panel.grid.major = element_blank(), panel.grid.minor = element_blank()) +
guides(fill=guide_legend(nrow=3, byrow=TRUE, color=guide_legend(nrow=3),reverse
  = TRUE, title.position="top"))+
xlab("")+ ylab("Mean Relative Abundance %")
p4
## plot together & save
tiff('CommunityAge.tiff', units="in", width=5, height=4, res=300, compression = 'lzw')
see::plots(p3,p4, n_columns = 1, tags=c("A", "B"))
dev.off()

#### Phylogenetic differences via heat trees of GIT sites ####
## following metacoder example analysis
library(metacoder)
library(taxa)

## be sure to set.seed to ensure the plots are the same if you go back to recreate
set.seed(9242)

## Convert rarified phyloseq object to taxmap
## subset between sites
Col<-subset_samples(phyb.rar, GIT=="Colon")
Je<-subset_samples(phyb.rar, GIT=="Jejunum")

##convert
tm_obj <- parse_phyloseq(Col)
jtm_obj <- parse_phyloseq(Je)

# get rid of low counts
tm_obj$data$tax_data <- zero_low_counts(tm_obj, data = "otu_table", min_count = 5)
jtm_obj$data$tax_data <- zero_low_counts(jtm_obj, data = "otu_table", min_count = 5)

##Check observations
cno_reads <- rowSums(tm_obj$data$tax_data[, , tm_obj$data$sample_data$sample_id])
== 0
sum(cno_reads)
jno_reads <- rowSums(jtm_obj$data$tax_data[, jtm_obj$data$sample_data$sample_id]) == 0
sum(jno_reads)

##remove
ctm_obj <- filter_obs(ctm_obj, data = "tax_data", ! cno_reads, drop_taxa = TRUE)
print(ctm_obj)

jtm_obj <- filter_obs(jtm_obj, data = "tax_data", ! jno_reads, drop_taxa = TRUE)
print(jtm_obj)

## calculate abundance
ctm_obj$data$tax_abund <- calc_taxon_abund(ctm_obj, "tax_data",
cols = ctm_obj$data$sample_data$sample_id)

jtm_obj$data$tax_abund <- calc_taxon_abund(jtm_obj, "tax_data",
cols = jtm_obj$data$sample_data$sample_id)

## counts per sample type
ctm_obj$data$tax_occ <- calc_n_samples(ctm_obj, "tax_abund", groups =
ctm_obj$data$sample_data$GIT, cols = ctm_obj$data$sample_data$sample_id)

jtm_obj$data$tax_occ <- calc_n_samples(jtm_obj, "tax_abund", groups =
jtm_obj$data$sample_data$GIT, cols = jtm_obj$data$sample_data$sample_id)

##plot
heat_tree(jtm_obj,
    node_label = ifelse(n_obs == 0,"", taxon_names),
    node_size = n_obs,
    node_color = Jejunum,
    node_size_axis_label = "ASV count",
    node_color_axis_label = "Samples with reads",
    node_color_range=c("grey74","khaki1","green", "deepskyblue" ),
    node_size_range = c(0.005, 0.03),
    edge_size_range=c(0.0005, 0.013),
    edge_label_size_range = c(10, 14),
    node_label_max = 200, edge_label_max =200,
    initial_layout = "re", layout = "da",
    output_file = "jejunum_heat_tree.pdf")

#### Colon
heat_tree(ctm_obj,
    node_label = ifelse(n_obs == 0,"", taxon_names),
    node_size = n_obs,
    node_color = Colon,
    node_size_axis_label = "ASV count",...
node_color_axis_label = "Samples with reads",
node_color_range=c("grey74","khaki1","green", "deepskyblue" ),
node_size_range = c(0.005, 0.03),
edge_size_range=c(0.0005, 0.013),
edge_label_size_range = c(10, 14),
initial_layout = "re", layout = "da",
output_file = "colon_heat_tree.pdf")

### Phylogenetic differences via heat trees of AgeClass ###

tm_obj <- parse_phyloseq(phyb.rar)

# get rid of low counts
tm_obj$data$tax_data <- zero_low_counts(tm_obj, data = "otu_table", min_count = 3)

## Check observations
no_reads <- rowSums(tm_obj$data$tax_data[, tm_obj$data$sample_data$sample_id]) == 0
sum(no_reads)
##259
## remove
tm_obj <- filter_obs(tm_obj, data = "tax_data", !no_reads, drop_taxa = TRUE)
print(tm_obj)

## counts
tm_obj$data$tax_abund <- calc_taxon_abund(tm_obj, "tax_data",
  cols = tm_obj$data$sample_data$sample_id)

## counts per ageclass
tm_obj$data$tax_occ <- calc_n_samples(tm_obj, "tax_abund", groups =
  tm_obj$data$sample_data$AgeClass, cols = tm_obj$data$sample_data$sample_id)
print(tm_obj)

## different
tm_obj$data$diff_table <- compare_groups(tm_obj,
  dataset = "tax_abund",
  cols = tm_obj$data$sample_id,
  groups = tm_obj$data$sample_data$AgeClass)# What columns of sample data to use
print(tm_obj$data$diff_table)

tm_obj <- mutate_obs(tm_obj, "diff_table",
  wilcox_p_value = p.adjust(wilcox_p_value, method = "fdr"))
tm_obj$data$diff_table$log2_median_ratio[tm_obj$data$diff_table$wilcox_p_value > 0.05] <- 0
range(tm_obj$data$diff_table$wilcox_p_value, finite = TRUE)
## no significant difference so no reason to move further

#### Bray NMDS Visualization ####

code:
```r
mds_bra<-metaMDS(beta_bra,distance="bray",k=4, trymax=1000, wascores = F)
```

#check out plot
plot(mds_bra)

# make a nicer plot
data.score<- as.data.frame(scores(mds_bra))
data.score$site<-rownames(data.score)
data.score$GIT<-alpha_table1$GIT
data.score$Sex<-alpha_table1$Sex
data.score$AgeClass<-alpha_table1$AgeClass
head(data.score)
data.score

GA <- data.score[data.score$GIT == "Jejunum", ][chull(data.score[data.score$GIT == "Jejunum", c("NMDS4", "NMDS3")]), ]
GB <- data.score[data.score$GIT == "Colon", ][chull(data.score[data.score$GIT == "Colon", c("NMDS4", "NMDS3")]), ]

## combine Groups
hull.data <- rbind(GA, GB)
tiff('NMDSbray.tiff', units="in", width=7, height=4, res=300, compression = 'lzw')
ggplot() +
  geom_polygon(data=hull.data,aes(x=NMDS4,y=NMDS3,group=GIT,
   fill=GIT),alpha=0.30) +
  geom_point(data=data.score,aes(x=NMDS4,y=NMDS3,colour=GIT,
   shape=AgeClass),size=2) +
  scale_shape_manual(values=c(1,2,4)) +
  scale_colour_manual(values=c("Jejunum" = "darkgreen", "Colon" = "cadetblue")) +
  scale_fill_manual(values=c("Jejunum" = "gray", "Colon" = "thistle3")) +
  coord_flip() +
  theme_bw() +
  theme( # remove y-axis text
    legend.position="bottom",
    axis.ticks = element_blank(),
    panel.grid.minor = element_blank(), # Vertical major grid lines
  )
```
```r
panel.grid.major = element_blank(), axis.text.x = element_text(family="Arial",size=8, color="black"), axis.text.y = element_text(family="Arial",size=8, color="black"), legend.text = element_text(size=6, family="Arial"), axis.title.x = element_text(family="Arial",size=8), axis.title.y = element_text(size=8, family="Arial"), panel.background = element_blank(), legend.title = element_blank(), plot.background = element_blank())+ ggtitle("Bray Curtis")
```

#### Jaccard NMDS Visualization ####

```r
mds_ja<-metaMDS(beta_ja,distance="jaccard",k=4, trymax=1000, wascores = F)
```

#check out plot

```r
plot(mds_ja)
```

# make a nicer plot

```r
data.score<- as.data.frame(scores(mds_ja))
data.score$site<-rownames(data.score)
data.score$GIT<-alpha_table1$GIT
data.score$Sex<-alpha_table1$Sex
data.score$AgeClass<-alpha_table1$AgeClass
```

```r
head(data.score)
data.score
```

```r
GA <- data.score[data.score$GIT == "Jejunum", ][chull(data.score[data.score$GIT == "Jejunum", c("NMDS4", "NMDS2")]), ]
```

```r
GB <- data.score[data.score$GIT == "Colon", ][chull(data.score[data.score$GIT == "Colon", c("NMDS4", "NMDS2")]), ]
```

## combine Groups

```r
hull.data <- rbind(GA, GB)
```

```r
tiff('NMDjacS.tiff', units="in", width=7, height=4, res=300, compression = 'lzw')
```

```r
ggplot() + geom_polygon(data=hull.data,aes(x=NMDS4,y=NMDS2,group=GIT, fill=GIT),alpha=0.30) + geom_point(data=data.score,aes(x=NMDS4,y=NMDS2,colour=GIT, shape=AgeClass),size=2) + scale_shape_manual(values=c(1,2,4)) + scale_colour_manual(values=c("Jejunum" = "darkgreen", "Colon" = "cadetblue")) + scale_fill_manual(values=c("Jejunum" = "gray", "Colon" = "thistle3")) + coord_flip() + theme_bw() + theme( legend.position="bottom",
```
axis.ticks = element_blank(),
panel.grid.minor = element_blank(),
panel.grid.major = element_blank(),
axis.text.x = element_text(family="Arial",size=8, color="black"),axis.text.y =
element_text(family="Arial",size=8, color="black"),
legend.text = element_text(size=8, family="Arial"),
axis.title.x = element_text(family="Arial",size=8),
axis.title.y = element_text(size=8, family="Arial"),
panel.background = element_blank(), legend.title = element_blank(),
plot.background = element_blank())+ ggtitle("Jaccard")
device.off()

#### weighted Unifrac Visualization ####

weighted<-phyloseq::ordinate(new_obj.rel, "PCoA", "unifrac", weighted=TRUE)

tiff('weighted.tiff', units="in", width=7, height=4, res=300, compression = 'lzw')
phyloseq::plot_ordination(new_obj.rel, weighted, color="GIT", shape="AgeClass")+
geom_point(size=2)+ scale_color_manual(values=c("cadetblue",
"darkgreen"))+scale_shape_manual(values=c(4,2,1))+
theme(
  legend.position="bottom",
  axis.ticks = element_blank(),
  axis.text.x = element_text(family="Arial",size=8, color="black"),axis.text.y =
element_text(family="Arial",size=8, color="black"),
  legend.text = element_text(size=8, family="Arial"),
  axis.title.x = element_text(family="Arial",size=8),
  axis.title.y = element_text(size=8, family="Arial"),
  panel.background = element_blank(), legend.title = element_blank(),
  plot.background = element_blank(), panel.border = element_rect(colour = "black",
  fill=NA, size=.5))+ggtitle("Weighted Unifrac")
device.off()

#### unweighted Unifrac Visualization ####

unweighted<-phyloseq::ordinate(new_obj, "PCoA", "unifrac", weighted=F)

tiff('unweighted.tiff', units="in", width=7, height=4, res=300, compression = 'lzw')
phyloseq::plot_ordination(new_obj, unweighted, color="GIT", shape="AgeClass")+
geom_point(size=2)+ scale_color_manual(values=c("cadetblue",
"darkgreen"))+scale_shape_manual(values=c(4,2,1))+
theme(
  legend.position="bottom",
  axis.ticks = element_blank(),
axis.text.x = element_text(family="Arial",size=8, color="black"), axis.text.y = element_text(family="Arial",size=8, color="black"),
legend.text = element_text(size=8, family="Arial"),
axis.title.x = element_text(family="Arial",size=8),
axis.title.y = element_text(size=8, family="Arial"),
panel.background = element_blank(), legend.title = element_blank(),
plot.background = element_blank(), panel.border = element_rect(colour = "black", fill=NA, size=.5))+ ggtitle("Unweighted Unifrac")
dev.off()

## heat trees of GIT sites
## follow the metacoder example analysis

library(metacoder)
library(taxa)

## be sure to set.seed to ensure the plots are the same if you go back to recreate
set.seed(9242)

## Convert rarified phyloseq object to taxmap
## subset between sites
Col<subset_samples(phyb.rar, GIT="Colon")
Je<subset_samples(phyb.rar, GIT="Jejunum")

## convert ctm_obj <- parse_phyloseq(Col)
jtm_obj <- parse_phyloseq(Je)

# get rid of low counts
cno_reads <- rowSums(ctm_obj$data$tax_data[, ctm_obj$data$sample_data$sample_id]) == 0
sum(cno_reads)
jno_reads <- rowSums(jtm_obj$data$tax_data[, jtm_obj$data$sample_data$sample_id]) == 0
sum(jno_reads)

## remove
ctm_obj <- filter_obs(ctm_obj, data = "tax_data", !cno_reads, drop_taxa = TRUE)
print(ctm_obj)

jtm_obj <- filter_obs(jtm_obj, data = "tax_data", !jno_reads, drop_taxa = TRUE)
print(jtm_obj)

## calculate abundance
c tm_obj$data$tax_abund <- calc_taxon_abund(ctm_obj, "tax_data",
cols = ctm_obj$data$sample_data$sample_id)

jtm_obj$data$tax_abund <- calc_taxon_abund(jtm_obj, "tax_data",
cols = jtm_obj$data$sample_data$sample_id)

## counts per sample type
c tm_obj$data$tax_occ <- calc_n_samples(ctm_obj, "tax_abund", groups =
c tm_obj$data$sample_data$GIT, cols = ctm_obj$data$sample_data$sample_id)

jtm_obj$data$tax_occ <- calc_n_samples(jtm_obj, "tax_abund", groups =
jtm_obj$data$sample_data$GIT, cols = jtm_obj$data$sample_data$sample_id)

print(tm_obj)

## uneven sampling
c tm_obj$data$tax_data <- calc_obs_props(ctm_obj, "tax_data")
jtm_obj$data$tax_data <- calc_obs_props(jtm_obj, "tax_data")

## plot
heat_tree(jtm_obj,
    node_label = ifelse(n_obs == 0,"", taxon_names),
    node_size = n_obs,
    node_color = Jejunum,
    node_size_axis_label = "ASV count",
    node_color_axis_label = "Samples with reads",
    node_color_range=c("grey74","khaki1","green", "deepskyblue" ),
    node_size_range = c(0.005, 0.03),
    edge_size_range=c(0.0005, 0.013),
    edge_label_size_range = c(10, 14),
    node_label_max = 200, edge_label_max =200,
    initial_layout = "re", layout = "da",
    output_file = "jejunum_heat_tree.pdf")

#### Colon
heat_tree(ctm_obj,
    node_label = ifelse(n_obs == 0,"", taxon_names),
    node_size = n_obs,
    node_color = Colon,
    node_size_axis_label = "ASV count",
    node_color_axis_label = "Samples with reads",
    node_color_range=c("grey74","khaki1","green", "deepskyblue" ),
    node_size_range = c(0.005, 0.03),
    edge_size_range=c(0.0005, 0.013),
    edge_label_size_range = c(10, 14),
    node_label_max = 200, edge_label_max =200,
    initial_layout = "re", layout = "da",
    output_file = "jejunum_heat_tree.pdf")
edge_label_size_range = c(10, 14),
initial_layout = "re", layout = "da",
output_file = "colon_heat_tree.pdf")

#### Boxplots of Faith's PD ####
tiff('PD.tiff', units="in", width=5, height=4, res=300, compression = 'lzw')
ggboxplot(alpha_table1, x = "AgeClass", y = "log", fill= "AgeClass", line.size = 0.4)+
scale_fill_manual(values=c("dark grey", "forestgreen", "cadetblue"))+
theme(legend.title = element_blank(), legend.text= element_text(size=9,
family="Arial"),
    axis.title.x = element_text(family="Arial",size=9), axis.text.x=
    element_text(family="Arial",size=9),axis.y= element_text(family="Arial",size=9),
    axis.title.y = element_text(size=9, family="Arial"))+
ylab("Faith's PD")+ xlab("")
dev.off()
APPENDIX I

SUPPLEMENTAL R SCRIPT FOR CHAPTER 2

#####CHAPTER TWO SUPPLEMENTAL R SCRIPT FOR GMB STATISTICAL ANALYSIS #####

library(microbiome) ## data analysis
library(qiime2R) # import data
library(phylloseq) # also the basis of data object. Data analysis and visualization
library(vegan) # some utility tools
library(data.table) # alternative to data.frame
library(dplyr) # data handling
library(tidyverse)
library(DT) ## interactive tables
library(ggpubr) ## plotting
library(ggplot2)
library(mctoolsr)
library(picante) ## faith's PD
library(see)
library(Rmisc)## graphing
library(picante)

setwd("~/Desktop/Projects/Bear/Bear-R/CLEAN/FINAL")

##### Import & create phylloseq dataframe with qiime2R and QIIME2 artifacts #####
##### Following Tutorial: Integrating QIIME2 and R for data visualization and analysis using qiime2R by J. Bisanz
##### you will need
# 1.) Metafile.tsv will need to have the second row removed and the # infront of SampleID removed for it to read okay
# 2.) taxonomy.qza
# 3.) table.qza
# 4.) rooted.qza

## import artifacts & metadata file
metadata<-
\read_tsv("Metafile.tsv")
SVs<-
\read_qza("table.qza")
taxonomy<-
\read_qza("taxonomy.qza")
taxtable<-taxonomy$data %>% as_tibble() %>% separate(Taxon, sep=";", c("Domain", "Phylum", "Class", "Order", "Family", "Genus", "Species")) #convert the table into a tabular split version
tree<-read_qza("rooted-tree.qza")

## Create the phyloseq object
phy_obj<-phyloseq(  
  otu_table(SVs$data, taxa_are_rows = T),  
  phy_tree(tree$data),  
  tax_table(as.data.frame(taxtable) %>% select(-Confidence) %>% column_to_rownames("Feature.ID") %>% as.matrix), #moving the taxonomy to the way phyloseq wants it  
  sample_data(metadata %>% as.data.frame() %>% column_to_rownames("SampleID")))

## view data table
datatable(tax_table(phy_obj))

##### Clean Taxonomy table ######
## Rename NAs to last known group
tax.clean <- data.frame(tax_table(phy_obj))
for (i in 1:7){ tax.clean[,i] <- as.character(tax.clean[,i])}
tax.clean[is.na(tax.clean)] <- ""

for (i in 1:nrow(tax.clean)){
  if (tax.clean[i,2] == ""){
    kingdom <- paste("Kingdom", tax.clean[i,1], sep = "")
    tax.clean[i, 2:7] <- kingdom
  } else if (tax.clean[i,3] == ""){
    phylum <- paste("Phylum", tax.clean[i,2], sep = "")
    tax.clean[i, 3:7] <- phylum
  } else if (tax.clean[i,4] == ""){
    class <- paste("Class", tax.clean[i,3], sep = "")
    tax.clean[i, 4:7] <- class
  } else if (tax.clean[i,5] == ""){
    order <- paste("Order", tax.clean[i,4], sep = "")
    tax.clean[i, 5:7] <- order
  } else if (tax.clean[i,6] == ""){
    family <- paste("Family", tax.clean[i,5], sep = "")
    tax.clean[i, 6:7] <- family
  } else if (tax.clean[i,7] == ""){
    tax.clean$Species[i] <- paste("Genus",tax.clean$Genus[i], sep = "_")
  }
}
## import new taxonomy table

tax_table(phy_obj) <- as.matrix(tax.clean)

## view
datatable(tax_table(phy_obj))

##### Rename uncultured

tax.clean2 <- data.frame(tax_table(phy_obj))

for (i in 1:7){ tax.clean2[,i] <- as.character(tax.clean2[,i])}
for (i in 1:nrow(tax.clean2)){
  if (tax.clean2[i,2] == "uncultured"){
    kingdom <- paste("Kingdom_", tax.clean2[i,1], sep = "")
    tax.clean2[i, 2:7] <- kingdom
  } else if (tax.clean2[i,3] == "uncultured"){
    phylum <- paste("Phylum_", tax.clean2[i,2], sep = "")
    tax.clean2[i, 3:7] <- phylum
  } else if (tax.clean2[i,4] == "uncultured"){
    class <- paste("Class_", tax.clean2[i,3], sep = "")
    tax.clean2[i, 4:7] <- class
  } else if (tax.clean2[i,5] == "uncultured"){
    order <- paste("Order_", tax.clean2[i,4], sep = "")
    tax.clean2[i, 5:7] <- order
  } else if (tax.clean2[i,6] == "uncultured"){
    family <- paste("Family_", tax.clean2[i,5], sep = "")
    tax.clean2[i, 6:7] <- family
  } else if (tax.clean2[i,7] == ""){
    tax.clean2$Species[i] <- paste("Genus",tax.clean2$Species[i], sep = " ")
  }
}

## import new taxonomy table

tax_table(phy_obj) <- as.matrix(tax.clean2)

## view new table
datatable(tax_table(phy_obj))

## import new taxonomy table

tax_table(phy_obj) <- as.matrix(tax.clean2)

## view new table
datatable(tax_table(phy_obj))

## save phyloseq object
saveRDS(phy_obj, "~/Desktop/Projects/Bear/Bear-R/CLEAN/FINAL/physeq.rds")

## if you ever want to pull back in
phy_obj<- readRDS("physeq.rds")

##### Alpha Diversity ####

## Equal sample sums
set.seed(9242) ## ensures rarifies the same each time script is run

summary(sample_sums(phy_obj)) ## helps determine depth for rarifying

## rarefying: we already know our depth: 1050 so rarefy to that
phyb.rar <- rarefy_even_depth(phy_obj, sample.size = 1050)
## lost one sample: S100J
summary(sample_sums(phyb.rar)) ## checking to see they all have the same sequence depth
any(taxa_sums(phy_obj)== 0) # making sure we dont have any sequences not in at least one samples
### run this is you do have 0's:ps1a <- prune_taxa(taxa_sums(phyb.rar) > 0, phyb.rar)

## pull metadata from physeq object
sam.meta <- meta(phyb.rar)
sam.meta

## put variables is particular order
sam.meta$GIT<-factor(sam.meta$GIT, levels=c("Jejunum", "Colon"))
sam.meta$AgeClass<-factor(sam.meta$AgeClass, levels=c("Yearling", "Subadult", "Adult", "Unknown"))

## Add the rownames as a new column for easy integration later.
sam.meta$sam_name <- rownames(sam.meta)

#### Richness: observed ASVs ####
# calculated with microbiome package
div_ch<- microbiome::alpha(new_obj, index="observed")

## Add the rownames to diversity table
div_ch$sam_name <- rownames(div_ch)

#### Phylogenetic diversity: Faith's PD ####
# Phylogenetic diversity is calculated using the picante package.

## pull ASV table
phyb.rar.asvtab <- as.data.frame(new_obj@otu_table)

## pull tree
phyb.rar.tree <- new_obj@phy_tree

## We first need to check if the tree is rooted or not
new_obj@phy_tree
###rooted so we are good to go
## Getting the data ready

div_pd <- pd(t((phyb.rar.asvtab), phyb.rar.tree, include.root=T))
# t(ou_table) transposes the table for use in picante and the
# tree file comes from the first code we used to read tree
# file (see making a phyloseq object section)

## Add the rownames to diversity table
div_pd$sam_name <- rownames(div_pd)

## STEP 4p. merge all of the alphas into one file
merged_table <- merge(div_pd, div_ch, by = "sam_name", all=T)
merged_table2 <- merge(merged_table, sam.meta, by = "sam_name", all=T)
alpha_table <- merge(merged_table2, div_shan, by = "sam_name", all=T)

datatable(alpha_table)  ## this now has all alpha measures in one datatable!

## add diet proportions to alpha table
attach(prop5)
alpha_table0 <- alpha_table

alpha_table0 <- merge(alpha_table0, prop5, by = "Subject", all=T)

datatable(alpha_table0)

#### LMER Analysis for Alpha diversity ####

library(lmerTest)
library(lme4)
library(car)
library(emmeans)
library(sjmisc)
library(sjPlot)
library(tidyverse)
library(wiqid)
library(MASS)

## remove unknown ages
alpha_table0 <- alpha_table

alpha_table0$Age[alpha_table0$Age==0] <- NA
alpha_table0$Age <- alpha_table0[complete.cases(alpha_table0$Age),]

alpha_table0$GIT <- factor(alpha_table0$GIT, levels=c("Jejunum", "Colon"))
alpha_table0$AgeClass <- factor(alpha_table0$AgeClass, levels = c("Yearling", "Subadult", "Adult"))

# This is so that distributions that must be non-zero can make sense of data
library(fitdistrplus)
descdist(alpha_table0$PD, discrete = FALSE, boot = 600)
fw <- fitdist(alpha_table0$PD, "weibull")
fg <- fitdist(alpha_table0$PD, "gamma")
fe <- fitdist(alpha_table0$PD, "exp")
fn <- fitdist(alpha_table0$PD, "lnorm")
n <- fitdist(alpha_table0$PD, "norm")
par(mfrow = c(1, 1))
plot.legend <- c("Weibull", "gamma", "expo", "lnorm")
denscomp(list(fn))
qqcomp(list(fn), legendtext = plot.legend)
cdfcomp(list(fn), legendtext = plot.legend)
ppcomp(list(fn), legendtext = plot.legend)
gofstat(list(fw, fg, fe))

#### LMER PD ####
## histogram
ggplot(alpha_table0, aes(x = PD)) + geom_histogram()
ggplot(alpha_table0, aes(x = log(PD))) + geom_histogram() + ggtitle("log transformed PD values")

## skewed right so log transform
?glmerControl()
## create initial models with maximum likelihood
pd_lme0 <- lme4::lmer(log(PD) ~ 1 + (1 | Subject), data = alpha_table0, REML = F)
pd_lme1 <- lme4::lmer(log(PD) ~ GIT*Human + (1 | Subject), data = alpha_table0, REML = F)
pd_lme2 <- lme4::lmer(log(PD) ~ GIT + Human + (1 | Subject), data = alpha_table0, REML = F)
pd_lme3 <- lme4::lmer(log(PD) ~ Bait + GIT + (1 | Subject), data = alpha_table0, REML = F)
pd_lme4 <- lme4::lmer(log(PD) ~ Bait + GIT + Corn + (1 | Subject), data = alpha_table0, REML = F)
pd_lme5 <- lme4::lmer(log(PD) ~ Human + (1 | Subject), data = alpha_table0, REML = F)
pd_lme6 <- lme4::lmer(log(PD) ~ Corn + (1 | Subject), data = alpha_table0, REML = F)
pd_lme7 <- lme4::lmer(log(PD) ~ Bait*GIT + (1 | Subject), data = alpha_table0, REML = F)
pd_lme8 <- lme4::lmer(log(PD) ~ Corn*GIT + (1 | Subject), data = alpha_table0, REML = F)
pd_lme9 <- lme4::lmer(log(PD) ~ Corn + GIT + (1 | Subject), data = alpha_table0, REML = F)
pd_lme10 <- lme4::lmer(log(PD) ~ Corn + AgeClass + (1 | Subject), data = alpha_table0, REML = F)
pd_lme11 <- lme4::lmer(log(PD) ~ Bait*AgeClass + (1 | Subject), data = alpha_table0, REML = F)
library(AICcmodavg)
model_list<-list(pd_lme0, pd_lme1,
pd_lme14,pd_lme13,pd_lme12,pd_lme11,pd_lme10,pd_lme2,pd_lme3,pd_lme5,
pd_lme4, pg_lme6, pd_lme7,pd_lme8,pd_lme9)
model_names<-c("NULL","model1","model14","model13","model12","model11","model10","model2","model3","model5","model4","model6","model7","model8","model9")
modelsel<-AICcmodavg::aictab(model_list, model_names, second.ord=T)
modelsel

## check distribution
##REML=T
pg_lme6<-lme4::lmer(log(PD)~Corn+(1|Subject),data=alpha_tabl0,REML = T)
performance::check_distribution(pg_lme6)
performance::check_model(pg_lme6)
performance::check_heteroscedasticity(pg_lme6, log(alpha_tabl0$PD))
##0.08
par(mfrow=c(1,1))
plot(pg_lme6)

## Assumption 2 Homogeneity of Variance
#Regression models assume that variance of the residuals
#is equal across groups.
#extracts the residuals and places them in a new column in our original data table
alpha_tabl0$lme10<- residuals(pg_lme6)
alpha_tabl0$baslme10 <-abs(alpha_tabl0$lme10) #creates a new column with the
absolute value of the residuals
alpha_tabl0$lme102 <- alpha_tabl0$baslme10^2 #squares the absolute values of the
residuals to provide the more robust estimate
pd_leven <- lm(lme102 ~ Subject, data=alpha_tabl0) #ANOVA of the squared residuals
anova(pd_leven) #displays the results
## good P=0.17

library(lme4)
library(rstan)
summary(Mod_lme6)
## run final model to get
confint.merMod(Mod_lme6, level=.95)
confint(Mod_lme6, level=.95)
Anova(pg_lme6)

## get $R^2$
library(performance)
performance::r2(pg_lme6)

## plot
library(extrafont)
loadfonts()
fonts()

p<-ggplot(alpha_table0, aes(x=Corn, y=PD)) +
  geom_point() +
  geom_smooth(method=lm, size=0.5, color="black") +
  theme(axis.text.x = element_text(color="black", family="Times New Roman", size=10),
        axis.text.y = element_text(color="black", family="Times New Roman", size=10),
        panel.background = element_blank(), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.title.x = element_text(family="Times New Roman"),
        axis.line = element_line(color="black"),
        strip.text = element_text(family="Times New Roman", size=12),
        strip.background = element_blank(),
        axis.title.y = element_text(family="Times New Roman"))) +
  ylab("Faith's PD") +
  xlab("Proportional Contribution of Corn %") +
  facet_wrap(~GIT)

ggsave("CornPD.pdf", p)

## Assumption 1 - Linearity

## Graphically, plotting the model residuals (the difference between the observed value and the model-estimated value) vs the predictor
alpha_table1<-alpha_table0
alpha_table1$log<-log(alpha_table1$PD)
Plot.Model.F.Linearity<-plot(resid(pg_lme6), alpha_table1$log)

## Assumption 2 Homogeneity of Variance
#Regression models assume that variance of the residuals is equal across groups.
# extracts the residuals and places them in a new column in our original data table
alpha_table1$lme10 <- residuals(Modpg_lme6)
alpha_table1$baslme10 <- abs(alpha_table1$lme10)  # creates a new column with the absolute value of the residuals
alpha_table1$lme102 <- alpha_table1$baslme10^2  # squares the absolute values of the residuals to provide the more robust estimate
pd_leven <- lm(lme102 ~ Subject, data=alpha_table1)  # ANOVA of the squared residuals
anova(pd_leven)  # displays the results

## visually
plot(pg_lme6)  # creates a fitted vs residual plot

## Assumption 3: The residuals of the model are normally distributed.

## QQ plots
library(lattice)

qqmath(pg_lme6, id=0.05)
## overall looks good!!

#### LMER Shannon ####
## histogram
ggplot(alpha_table0,aes(x=diversity_shannon))+geom_histogram()
## need to log transformed

## create intial models with maximum likelihood
sh_lme0 <- lme4::lmer(diversity_shannon~1+(1|Subject), data=alpha_table0, REML = F)
sh_lme1 <- lme4::lmer(diversity_shannon~GIT*Human+(1|Subject),data=alpha_table0,REML = F)
sh_lme2 <- lme4::lmer(diversity_shannon~GIT+Human+(1|Subject),data=alpha_table0,REML = F)
sh_lme3 <- lme4::lmer(diversity_shannon~Bait+GIT+(1|Subject),data=alpha_table0,REML = F)
sh_lme4 <- lme4::lmer(diversity_shannon~Bait+GIT+Corn+(1|Subject),data=alpha_table0,REML = F)
sh_lme5 <- lme4::lmer(diversity_shannon~Human+(1|Subject),data=alpha_table0,REML = F)
sh_lme6 <- lme4::lmer(diversity_shannon~Corn+(1|Subject),data=alpha_table0,REML = F)
sh_lme7 <- lme4::lmer(diversity_shannon~Bait*GIT+(1|Subject),data=alpha_table0,REML = F)
sh_lme8 <- lme4::lmer(diversity_shannon~Corn*GIT+(1|Subject),data=alpha_table0,REML = F)
sh_lme9 <- lme4::lmer(diversity_shannon~Corn+GIT+(1|Subject),data=alpha_table0,REML = F)
sh_lme10<- lme4::lmer(diversity_shannon~Corn+AgeClass+(1|Subject),data=alpha_tabl0,REML = F)
sh_lme11<- lme4::lmer(diversity_shannon~Bait*AgeClass+(1|Subject),data=alpha_tabl0,REML = F)
sh_lme12<- lme4::lmer(diversity_shannon~Bait+AgeClass+(1|Subject),data=alpha_tabl0,REML = F)
sh_lme13<- lme4::lmer(diversity_shannon~Human*AgeClass+(1|Subject),data=alpha_tabl0,REML = F)
sh_lme14<- lme4::lmer(diversity_shannon~Human+AgeClass+(1|Subject),data=alpha_tabl0,REML = F)

model_list<-list(sh_lme0, sh_lme1, sh_lme2,sh_lme3,sh_lme4,sh_lme5,sh_lme6,sh_lme7,sh_lme8,sh_lme9,sh_lme10, sh_lme11,sh_lme12,sh_lme13, sh_lme14)
model_names<- c("NULL","model1","model2","model3","model4","model5","model6","model7","model 8","model9","model10","model11","model12","model13","model14")
modelsel<-AICcmodavg::aictab(model_list, model_names, second.ord=T)

## check distribution
sh_lme6<-lme4::lmer(diversity_shannon~Corn+(1|Subject),data=alpha_tabl0,REML = T)
performance::check_distribution(sh_lme6)

performance::check_model(sh_lme0)
performance::check_heteroscedasticity(sh_lme6)
##.28
par(mfrow=c(1,1))
plot(pg_lme6)

## Assumption 2 Homogeneity of Variance
#Regression models assume that variance of the residuals
#is equal across groups.

#extracts the residuals and places them in a new column in our original data table
alpha_tabl0$lme10<- residuals(sh_lme6)
alpha_tabl0$baslme10 <- abs(alpha_tabl0$lme10) # creates a new column with the absolute value of the residuals
alpha_tabl0$lme102 <- alpha_tabl0$baslme10^2 # squares the absolute values of the residuals to provide the more robust estimate
pd_leven <- lm(lme102 ~ Subject, data=alpha_tabl0) # ANOVA of the squared residuals
anova(pd_leven) # displays the results
## good P=0.24

?exp()

summary(pg_lme6)
(exp(-2.2-0.9)-1)*100
## run final model to get
Anova(sh_lme6)

## get R^2
library(performance)
performance::r2(sh_lme6)

##plot
ggplot(alpha_tabl0, aes(x=Corn, y=log(PD))) +
  geom_point()+
  geom_smooth(method=lm)

## Assumption 1 - Linearity

## Graphically, plotting the model residuals (the difference # between the observed value and the model-estimated value) vs # the predictor
alpha_table1<-alpha_tabl0
Plot.Model.F.Linearity<-plot(resid(sh_lme6),alpha_table1$diversity_shannon)

## Assumption 2 Homogeneity of Variance
# Regression models assume that variance of the residuals # is equal across groups.

# extracts the residuals and places them in a new column in our original data table
alpha_table1$lme10<- residuals(sh_lme6)
alpha_table1$baslme10 <- abs(alpha_table1$lme10) # creates a new column with the absolute value of the residuals
alpha_table1$lme102 <- alpha_table1$baslme10^2 # squares the absolute values of the residuals to provide the more robust estimate
fs_leven <- lm(lme102 ~ Subject, data=alpha_table1) #ANOVA of the squared residuals
anova(fs_leven) #displays the results

## visually
plot(sh_lme6) #creates a fitted vs residual plot

## Assumption 3: The residuals of the model are normally distributed.

# QQ plots

qqmath(sh_lme6, id=0.05)
## little wonky!!

#### LMER Observed ASVs ####
# This is so that distributions that must be non-zero can make sense of data
alpha_table$observed.t <- alpha_table$observed
qqp(alpha_table$observed.t, "norm")

# lnorm means lognormal
qqp(alpha_table$observed.t, "lnorm")
## everything in CI
## run GLMM
## histogram

## gamma
gamma <- fitdistr(alpha_table$observed.t, "gamma")
qqp(alpha_table$observed.t, "gamma", shape = gamma$estimate[[1]], rate =
gamma$estimate[[2]])

## poisson
poisson <- fitdistr(alpha_table$observed.t, "Negative Binomial")
qqp(alpha_table$observed.t, "nbinom", size = poisson$estimate[[1]], mu =
poisson$estimate[[2]])
## looks great

ggplot(alpha_table0,aes(x=observed))+geom_histogram()
ggplot(alpha_table0,aes(x=log( observed)))+geom_histogram()+ ggtitle("log transformed PD values")

## skewed right so log transform
Mod_lme0<-glmer.nb(observed~1+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme1<-glmer.nb(observed~GIT*Human+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme2<-glmer.nb(observed~GIT+Human+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme3<-glmer.nb(observed~Bait+GIT+Corn+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme4<-glmer.nb(observed~Bait*GIT+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme5<-glmer.nb(observed~Human+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme6<-glmer.nb(observed~Corn+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme7<-glmer.nb(observed~Bait*GIT+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme8<-glmer.nb(observed~Corn*GIT+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))
Mod_lme9<- glmer.nb(observed~Corn+GIT+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme10<- glmer.nb(observed~Corn+AgeClass+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw", optCtrl=list(maxfun=2e5)))

Mod_lme11<- glmer.nb(observed~Bait*AgeClass+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw",optCtrl=list(maxfun=2e5)))

Mod_lme12<- glmer.nb(observed~Bait+AgeClass+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw",optCtrl=list(maxfun=2e5)))

Mod_lme13<- glmer.nb(observed~Human*AgeClass+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw",optCtrl=list(maxfun=2e5)))

Mod_lme14<- glmer.nb(observed~Human+AgeClass+(1|Subject),data=alpha_tabl0,nAGQ=0,control=glmerControl(optimizer="nmkbw",optCtrl=list(maxfun=2e5)))

model_list<-list(Mod_lme0, Mod_lme1, Mod_lme2,Mod_lme3,Mod_lme4,Mod_lme5,Mod_lme6,Mod_lme7,Mod_lme8, Mod_lme9, Mod_lme10, Mod_lme11,Mod_lme12,Mod_lme13, Mod_lme14)
model_names<-c("NULL","model1","model2","model3","model4","model5","model6","model7","model 8","model9","model10","model11","model12","model13","model14")
modelsel<-AICcmodavg::aictab(model_list, model_names, second.ord=T)
modelsel

## Assumption 2 Homogeneity of Variance
Regression models assume that variance of the residuals is equal across groups.

extracts the residuals and places them in a new column in our original data table

```r
alpha_tabl0$lme10 <- residuals(Mod_lme6)
alpha_tabl0$baslme10 <- abs(alpha_tabl0$lme10) # creates a new column with the absolute value of the residuals
alpha_tabl0$lme102 <- alpha_tabl0$baslme10^2 # squares the absolute values of the residuals to provide the more robust estimate
pd_leven <- lm(lme102 ~ Subject, data=alpha_tabl0) # ANOVA of the squared residuals
anova(pd_leven) # displays the results
## good P = 0.36
```

summary(Mod_lme6)
## run final model to get
Anova(Mod_lme6)
## Chisq Df Pr(>Chisq)
## Corn 5.63 1 0.01766 *

## get R^2
library(performance)
performance::r2(Mod_lme6)

Anova(Mod_lme6)
## Chisq Df Pr(>Chisq)
## Corn 5.63 1 0.01766 *

library(performance)
performance::r2(Mod_lme6)

anova(pd_leven) # displays the results
## good P = 0.36

## get R^2

##plot
extrafont::font_import()
pdf("CornObserved.pdf")
P1 <- ggplot(alpha_tabl0, aes(x=Corn, y=observed)) +
  geom_point() +
  geom_smooth(method=lm, size=0.5, color="black") +
  theme(axis.text.x = element_text(color="black", family="Times New Roman", size=10), axis.text.y = element_text(color="black", family="Times New Roman", size=10),
        panel.background = element_blank(), panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        axis.title.x = element_text(family="Times New Roman"), axis.line =
        element_line(color="black"), strip.text = element_text(family="Times New Roman", size=12),
        strip.background = element_blank(), axis.title.y = element_text(family="Times New Roman") +
        ylab("Observed ASVs") +
        xlab("Proportional Contribution of Corn %") +
        facet_wrap(~GIT)

ggsave("CornObserved.pdf", P1)
#### weighted unifrac

```r
pseq.rel <- microbiome::transform(phyb.rar, "compositional")
new_obj.rel= subset_samples(new_obj, AgeClass != "Unknown")

unifrac.dist <- UniFrac(new_obj.rel,
                        weighted = TRUE,
                        normalized = TRUE,
                        parallel = FALSE,
                        fast = TRUE)

WU_permanova <- adonis(unifrac.dist~ Bait+GIT+Corn+AgeClass,
                        strata=alpha_tabl0$Subject,data = alpha_tabl0)
WU_permanova2 <- adonis(unifrac.dist~ Bait*GIT+Corn,
                        strata=alpha_tabl0$Subject,data = alpha_tabl0)
WU_permanova3 <- adonis(unifrac.dist~ Bait+GIT*Corn,
                        strata=alpha_tabl0$Subject,data = alpha_tabl0)

WU_permanova3 <- adonis(unifrac.dist~ Corn*GIT+Bait,
                        strata=alpha_tabl0$Subject,data = alpha_tabl0)

WU_permanova3
```

#GIT

```r
permutest(betadisper(unifrac.dist, alpha_tabl0$Corn))
## not significant!
permutest(betadisper(unifrac.dist, alpha_tabl0$Bait))
## not significant
```

## unweighted

```r
new_obj= subset_samples(phyb.rar, AgeClass != "Unknown")
ununifrac.dist <- UniFrac(new_obj,
                          weighted = FALSE,
                          parallel = FALSE,
                          fast = TRUE)

unWU_permanova <- adonis(ununifrac.dist ~ Corn*GIT+Bait,
                        strata=alpha_tabl0$Subject,data = alpha_tabl0, na.rm=T)
unWU_permanova

permutest(betadisper(ununifrac.dist, alpha_tabl0$Corn))
```
## not significant!
permutest(betadisper(ununifrac.dist, alpha_table$GIT))

#### weighted Unifrac Visualization ####
weighted <- phyloseq::ordinate(new_obj.rel, "PCoA", "unifrac", weighted=TRUE)

tiff('weighted.tiff', units="in", width=7, height=4, res=300, compression = 'lzw')
phyloseq::plot_ordination(new_obj, unweighted, color="Corn", shape="GIT") +
  geom_point(size=2) + scale_color_continuous(type = "viridis",
  breaks=c(0, 1, 2, 3, 4, 5, 6), limits=c(0, 6)) +
  theme(
    legend.position="bottom",
    axis.ticks = element_blank(),
    axis.text.x = element_text(family="Times New Roman", size=8, color="black"),
    axis.text.y = element_text(family="Times New Roman", size=8, color="black"),
    legend.text = element_text(size=8, family="Times New Roman"),
    axis.title.x = element_text(family="Times New Roman", size=8),
    axis.title.y = element_text(family="Times New Roman"),
    panel.background = element_blank(), legend.title = element_blank(),
    plot.background = element_blank(), panel.border = element_rect(colour = "black",
    fill=NA, size=.5)) + ggtitle("")
dev.off()

#### unweighted Unifrac Visualization ####

devtools::install_github("jaredhuling/jcolors")
library(jcolors)

unweighted <- phyloseq::ordinate(new_obj, "PCoA", "unifrac", weighted=F)
tiff('unweighted.tiff', units="in", width=7, height=4, res=300, compression = 'lzw')
phyloseq::plot_ordination(new_obj.rel, weighted, color="Bait", shape="GIT") +
  geom_point(size=2) + scale_color_jcolors_continuous("pal4", reverse = TRUE, bias = 2.25) +
  theme(
    legend.position="bottom",
    axis.ticks = element_blank(),
    axis.text.x = element_text(family="Times New Roman", size=8, color="black"),
    axis.text.y = element_text(family="Times New Roman", size=8, color="black"),
    legend.text = element_text(size=8, family="Times New Roman"),
    axis.title.x = element_text(family="Times New Roman", size=8),
    axis.title.y = element_text(family="Times New Roman"),
)
```r
sample_data(new_obj)$Corn <- alpha_tabl0$Corn
sample_data(new_obj)$Bait <- alpha_tabl0$Bait

tiff("d13Faith.tif", res = 300, height = 4, width = 4, units = 'in')
ggplot(alpha_tabl0, aes(x=d13C, y=PD)) + geom_point() + geom_smooth(method=lm,size=0.5, color="black")+
theme(axis.text.x = element_text(color="black", family="Times New Roman", size=10),axis.text.y = element_text(color="black", family="Times New Roman", size=10),
panel.background=element_blank(),panel.grid.major = element_blank(),
axis.title.x = element_text(family="Times New Roman"),strip.text =element_text(family="Times New Roman", size=12), axis.line = element_line(color="black"),
strip.background = element_blank(),axis.title.y = element_text(family="Times New Roman")) +
xlab(expression(paste(delta^13,"C(\u2030)"))) + ylab("Faith's PD") + facet_wrap(~GIT)
dev.off()

tiff("d13ob.tif", res = 1200, height = 5, width = 5, units = 'in')
ggplot(alpha_tabl0, aes(x=d13C, y=observed)) + geom_point() + theme_classic() + geom_smooth(method=lm,size=0.5, color="black") + facet_wrap(~GIT+)
theme(axis.text.x = element_text(color="black", family="Times New Roman", size=10),axis.text.y = element_text(color="black", family="Times New Roman", size=10),
panel.background=element_blank(),panel.grid.major = element_blank(),
axis.title.x = element_text(family="Times New Roman"),strip.text =element_text(family="Times New Roman", size=12), axis.line = element_line(color="black"),
strip.background = element_blank(),axis.title.y = element_text(family="Times New Roman")) +
xlab(expression(paste(delta^13,"C(\u2030)"))) + ylab("Observed ASVs")
dev.off()
```
### CUSTOMIZED BIPLYT ###

#### customize Biplot from MixSIAR ####
#### these functions are modified from the original plot_data function provided by MixSIAR

```r
library(MixSIAR)
library(tidyr)
library(R2jags)
library(ggplot2)

plot_data_two_iso2 <-
function(isotopes,mix,source,discr,filename,plot_save_pdf,plot_save_png){
  # added only to pass R CMD check
  x <- y <- ymin <- ymax <- scolour <- xmin <- xmax <- label <- NULL

  # Plot the 2 input isotopes (iso1 on x-axis, iso2 on y-axis)
  df <- data.frame(x = mix$data_iso[,isotopes[1]], y = mix$data_iso[,isotopes[2]])
  # Look in the isotope column headers for 'C', 'N', 'S', and 'O'
  # Make the x and y labels for the isospace plot
  if(length(grep("C",mix$iso_names[isotopes[1]]))==1) x_label <-
    expression(paste(delta^13, "C (\u2030)",sep=""), family="Times New Roman")
  if(length(grep("N",mix$iso_names[isotopes[1]]))==1) x_label <-
    expression(paste(delta^15, "N (\u2030)",sep=""), family="Times New Roman")
  if(length(grep("S",mix$iso_names[isotopes[1]]))==1) x_label <-
    expression(paste(delta^34, "S (\u2030)",sep=""))
  if(length(grep("O",mix$iso_names[isotopes[1]]))==1) x_label <-
    expression(paste(delta^18, "O (\u2030)",sep=""))
  if(length(grep("SP",mix$iso_names[isotopes[1]]))==1) y_label <-
    expression(paste(delta^15, "N-SP (\u2030)",sep=""))
  if(length(grep("C",mix$iso_names[isotopes[2]]))==1) y_label <-
    expression(paste(delta^13, "C (\u2030)",sep=""))
  if(length(grep("N",mix$iso_names[isotopes[2]]))==1) y_label <-
    expression(paste(delta^15, "N (\u2030)",sep=""))
  if(length(grep("S",mix$iso_names[isotopes[2]]))==1) y_label <-
    expression(paste(delta^34, "S (\u2030)",sep=""))
  if(length(grep("O",mix$iso_names[isotopes[2]]))==1) y_label <-
    expression(paste(delta^18, "O (\u2030)",sep=""))
  if(length(grep("SP",mix$iso_names[isotopes[2]]))==1) y_label <-
    expression(paste(delta^15, "N-SP (\u2030)",sep=""))
  if(!exists("x_label")) x_label <- mix$iso_names[isotopes[1]]
  if(!exists("y_label")) y_label <- mix$iso_names[isotopes[2]]

  # Make the output filename
  if(plot_save_pdf) name <- paste("./figures/", discr, "_", source, "_.pdf", sep="")
  if(plot_save_png) name <- paste("./figures/", discr, "_", source, "_.png", sep="")

  # Plot the data
  df$x <- df$x - mean(df$x)
  df$y <- df$y - mean(df$y)
  df$x <- df$x / sd(df$x)
  df$y <- df$y / sd(df$y)

  ggplot(df, aes(x=x, y=y)) +
    geom_point(color=scolour, size=3) +
    geom_abline(intercept=0, slope=1, colour="blue") +
    xlab(x_label) + ylab(y_label) +
    theme(axis.title.x = element_text(family="Times New Roman")) +
    theme(axis.title.y = element_text(family="Times New Roman")) +
    theme(axis.title.x=element_text(family="Times New Roman"), size=10) +
    theme(axis.title.y=element_text(family="Times New Roman"), size=10) +
    theme(plot.title=element_text(family="Times New Roman"), size=12) +
    theme(plot.subtitle=element_text(family="Times New Roman"), size=10) +
    theme(plot.caption=element_text(family="Times New Roman"), size=8)
}
```
if(!is.na(source$by_factor)){
  source_linetype <- sort(rep(1:source$n.sources,source$S_factor_levels))  # each source gets a different linetype (assumes source$S_MU is sorted by source and then factor, which it is)
  source_color <- factor(as.numeric(source$S_factor1))  # color sources by factor 1 (ex: region)
  index <- seq(from=1,to=1+(source$n.sources-1)*source$S_factor_levels,by=source$S_factor_levels)  # "index" gets the row in source$S_MU of the first instance of each source (for making the source labels)
  discr_mu_plot <- array(NA,dim=c(length(source$S_MU[,1]),mix$n.iso))  # Since discr$mu is not by factor, it needs to be expanded out by 'source$S_factor_levels' to match the dimensions of source$S_MU. I.e. if source$n.sources=10, n.iso=2, and source$S_factor_levels=3 (condor data), frac_mu is 10x2 and source$S_MU is 30x2. This makes frac_mu_plot, a 30x2 matrix.
  discr_sig2_plot <- array(NA,dim=c(length(source$S_MU[,1]),mix$n.iso))  # Same for discr$sig2
  for(i in 1:source$n.sources){
    discr_mu_plot[index[i]:index[i]+source$S_factor_levels-1],] <- matrix(rep(discr$mu[i,],source$S_factor_levels),nrow=source$S_factor_levels,ncol=mix$n.iso,byrow=T)
    discr_sig2_plot[index[i]:index[i]+source$S_factor_levels-1],] <- matrix(rep(discr$sig2[i,],source$S_factor_levels),nrow=source$S_factor_levels,ncol=mix$n.iso,byrow=T)
  }
} else {  # source$by_factor==FALSE
  # each source gets a different linetype
  source_color <- factor(rep("black",source$n.sources))  # this doesn't work...solution was to make separate ggplot calls for by_factor and not_by_factor
  index <- 1:source$n.sources  # "index" gets the row in S_MU of the first instance of each source (since not by factor, only one instance of each source)
  discr_mu_plot <- discr$mu
  discr_sig2_plot <- discr$sig2
}

MU_plot <- array(NA,dim=c(length(source$S_MU[,1]),2))  # MU_plot will hold the source means adjusted for fractionation/enrichment
SIG_plot <- array(NA,dim=c(length(source$S_SIG[,1]),2))  # SIG_plot will hold the source sds adjusted for fractionation/enrichment
#for(src in 1:source$n.sources){
#  for(iso in 1:2){
#    MU_plot[,iso] <- source$S_MU[,isotopes[iso]] + discr_mu_plot[,isotopes[iso]]  # add fractionation mean to the source mean values
#    SIG_plot[,iso] <- sqrt(source$S_SIG[,isotopes[iso]]^2 + discr_sig2_plot[,isotopes[iso]])  # add fractionation sd to the source sd values
#  }
#}
df_sources <- data.frame(x=MU_plot[,1], y=MU_plot[,2],
  ymin = MU_plot[,2] - SIG_plot[,2],
  ymax = MU_plot[,2] + SIG_plot[,2],
  xmin = MU_plot[,1] - SIG_plot[,1],
  xmax = MU_plot[,1] + SIG_plot[,1],
  linetype = 1,
  scolour = source_color)

source.labels <- data.frame(
  x = MU_plot[index,1] - rep(1,source$n.sources), # label sources just left
  y = MU_plot[index,2] + rep(0.75,source$n.sources), # and up from their means
  label = source$source_names
)

.e <- environment()

develop()

eval()

eval()

eval()

eval()

eval()

eval()

eval()

eval()

eval()

eval()

eval()

eval()

eval()

eval()
ggplot2::xlab(x_label) +
ggplot2::theme(legend.position=c(0,1), legend.justification=c(0,1),
legend.title=ggplot2::element_blank(),
axis.text.x = element_text(color="black",
family="Times New Roman", size=10),
axis.text.y = element_text(color="black",
family="Times New Roman", size=10),
panel.background=element_blank(),
panel.grid.major = element_blank(),
panel.grid.minor = element_blank(),
axis.title.x = element_text(family="Times New Roman"),
axis.line = element_line(color="black"),
strip.background = element_blank(),
axis.title.y = element_text(family="Times New Roman"))

print(g)

} else { # sources not by factor (make the sources black)
g <- ggplot2::ggplot(data = df, ggplot2::aes(x = x, y = y), environment=.e) +
ggplot2::geom_point(ggplot2::aes(colour = factor(mix$FAC[[1]]$values),
shape = factor(mix$FAC[[2]]$values)),
size=2.5, show.legend=T) + # Factor.1

Factor.2
  ggplot2::scale_colour_discrete(breaks = levels(factor(mix$FAC[[1]]$values)),
  size=2.5, show.legend=T) + # Factor.1
labels = mix$FAC[[1]]$labels) + # factor1_names
  ggplot2::scale_shape_manual(values=shapes, labels=mix$FAC[[2]]$labels) + # factor2_names
  ggplot2::geom_pointrange(data=df_sources,
    ggplot2::aes(ymin=ymin, ymax=ymax),
    size=.5,
    linetype=1,
    show.legend=F) +
  ggplot2::geom_errorbarh(data=df_sources,
    ggplot2::aes(xmin=xmin, xmax=xmax),
    size=.5,
    height=0,
    linetype=1,
    show.legend=F) +
  ggplot2::geom_text(data=source.labels, position = position_nudge(x = -0.2),
    ggplot2::aes(x=x, y=y, label=label), show.legend=F, family="Times New Roman") +
  ggplot2::ylab(y_label) +
  ggplot2::xlab(x_label) +
  ggplot2::theme(legend.position=c(0,1), legend.justification=c(0,1),
legend.title=ggplot2::element_blank(),
axis.text.x = element_text(color="black",
family="Times New Roman", size=10),
axis.text.y = element_text(color="black",
family="Times New Roman", size=10),
panel.background=element_blank(),
panel.grid.major = element_blank(),
panel.grid.minor = element_blank(),
axis.title.x = element_text(family="Times New Roman"),
axis.line = element_line(color="black"),

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n.effects==2
}
if(mix$n.effects==1){
  if(!is.na(source$by_factor)){ # sources by factor, want to color the sources by factor1
    g <- ggplot2::ggplot(data = df, ggplot2::aes(x = x, y = y), environment=.e) +
    ggplot2::geom_point(ggplot2::aes(colour = factor(mix$FAC[[1]]$values)),
    show.legend=T) + # Factor.1
    ggplot2::scale_colour_discrete(breaks = levels(factor(mix$FAC[[1]]$values)),
    labels = mix$FAC[[1]]$labels) + # factor1_names
    ggplot2::geom_pointrange(data=df_sources,
    ggplot2::aes(ymin=ymin,ymax=ymax,colour=scolour),
    size=.5,
    linetype=1,
    show.legend=F) +
    ggplot2::geom_errorbarh(data=df_sources,
    ggplot2::aes(xmin=xmin,xmax=xmax,colour=scolour),
    size=.5,
    height=0,
    linetype=1,
    show.legend=F) +
    ggplot2::geom_text(data=source.labels, position = position_nudge(x = -0.2),
    ggplot2::aes(x=x,y=y,label=label), show.legend=F, family="Times New Roman",
    size=10) +
    ggplot2::ylab(y_label) +
    ggplot2::xlab(x_label) +
    ggplot2::theme(legend.position=c(0,1), legend.justification=c(0,1),
    legend.title=ggplot2::element_blank(),axis.text.x = element_text(color="black",
    family="Times New Roman", size=10),axis.text.y = element_text(color="black",
    family="Times New Roman", size=10),
    panel.background=element_blank(),panel.grid.major = element_blank(),
    panel.grid.minor = element_blank(),
    axis.title.x = element_text(family="Times New Roman"), axis.line =
    element_line(color="black"),
    strip.background = element_blank(),axis.title.y = element_text(family="Times New Roman"))
  }
}
else { # sources not by factor (make the sources black)
  g <- ggplot2::ggplot(data = df, ggplot2::aes(x = x, y = y), environment=.e) +
    ggplot2::geom_point(ggplot2::aes(colour = factor(mix$FAC[[1]]$values)),
    show.legend=T) + # Factor.1
    ggplot2::geom_pointrange(data=df_sources,
    ggplot2::aes(ymin=ymin,ymax=ymax,colour=scolour),
    size=.5,
    linetype=1,
    show.legend=F) +
    ggplot2::geom_errorbarh(data=df_sources,
    ggplot2::aes(xmin=xmin,xmax=xmax,colour=scolour),
    size=.5,
    height=0,
    linetype=1,
    show.legend=F) +
    ggplot2::geom_text(data=source.labels, position = position_nudge(x = -0.2),
    ggplot2::aes(x=x,y=y,label=label), show.legend=F, family="Times New Roman",
    size=10) +
    ggplot2::ylab(y_label) +
    ggplot2::xlab(x_label) +
    ggplot2::theme(legend.position=c(0,1), legend.justification=c(0,1),
    legend.title=ggplot2::element_blank(),axis.text.x = element_text(color="black",
    family="Times New Roman", size=10),axis.text.y = element_text(color="black",
    family="Times New Roman", size=10),
    panel.background=element_blank(),panel.grid.major = element_blank(),
    panel.grid.minor = element_blank(),
    axis.title.x = element_text(family="Times New Roman"), axis.line =
    element_line(color="black"),
    strip.background = element_blank(),axis.title.y = element_text(family="Times New Roman"))
  }
}

print(g)
ggplot2::scale_colour_discrete(breaks = levels(factor(mix$FAC[[1]]$values)), 
Factor.1
   labels = mix$FAC[[1]]$labels) + # factor1_names
ggplot2::geom_pointrange(data = df_sources,
   ggplot2::aes(ymin=ymin,ymax=ymax),
   size=0.5,
   linetype=1,
   show.legend=F) +
ggplot2::geom_errorbarh(data = df_sources,
   ggplot2::aes(xmin=xmin,xmax=xmax),
   size=0.5,
   height=0,
   linetype=1,
   show.legend=F) +
ggplot2::geom_text(data = source.labels,position = position_nudge(x = -0.2),
   ggplot2::aes(x=x,y=y,label=label), show.legend=F, family="Times New Roman",
   size=10) +
ggplot2::ylab(y_label) +
ggplot2::xlab(x_label) +
ggplot2::theme(text=element_text(family="Times New
   Roman"),legend.position=c(0,1), legend.justification=c(0,1),
   legend.title=ggplot2::element_blank(),axis.text.x = element_text(color="black",
   family="Times New Roman", size=10),axis.text.y = element_text(color="black",
   family="Times New Roman", size=10),
   panel.background=element_blank(),panel.grid.major = element_blank(),
   panel.grid.minor = element_blank(),
   axis.title.x = element_text(family="Times New Roman"), axis.line =
   element_line(color="black"),
   strip.background = element_blank(),axis.title.y = element_text(family="Times
   New Roman"))
print(g)
}
} # end n.effects==1
if(mix$n.effects==0){
g <- ggplot2::ggplot(data = df,ggplot2::aes(x = x,y = y)) +
ggplot2::geom_point() +
ggplot2::geom_pointrange(data=df_sources,
   ggplot2::aes(ymin=ymin,ymax=ymax),
   size=0.5,
   linetype=1,
   show.legend=F) +
ggplot2::geom_errorbarh(data=df_sources,
   ggplot2::aes(xmin=xmin,xmax=xmax),
   size=0.5,
   height=0,
   linetype=1,
show.legend=F) +
ggplot2::geom_text(data=source.labels, position = position_nudge(x = - 0.2),ggplot2::aes(x=x,y=y,label=label), show.legend=F, family="Times New Roman") +
ggplot2::ylab(y_label) +
ggplot2::xlab(x_label) +
ggplot2::theme(legend.position=c(0,1), legend.justification=c(0,1),
legend.title=ggplot2::element_blank(),axis.text.x = element_text(color="black",
family="Times New Roman", size=10),axis.text.y = element_text(color="black",
family="Times New Roman", size=10),
panel.background=element_blank(),panel.grid.major = element_blank(),
panel.grid.minor = element_blank(),
axis.title.x = element_text(family="Times New Roman"), axis.line =
 element_line(color="black"),
strip.background = element_blank(),axis.title.y = element_text(family="Times New Roman"))

print(g)
}
if(plot_save_pdf==TRUE){
mypath <-
file.path(paste(getwd(), "/", filename, "_", isotopes[1], "_", isotopes[2], ".pdf", sep=""))
# dev.copy2pdf(file=mypath)
cairo_pdf(filename=mypath, width=7, height=7)
print(g)
dev.off()
}
if(plot_save_png==TRUE){
mypath <-
file.path(paste(getwd(), "/", filename, "_", isotopes[1], "_", isotopes[2], ".png", sep=""))
png(filename=mypath,
    width= 7,
    height= 5,
    units= "in",
    res= 1200,
    pointsize = .5)
par(
    mar = c(5, 5, 2, 2),
    xaxs = "i",
    yaxs = "i",
    cex.axis = .5,
    cex.lab = .5)
print(g)
dev.off()
}
# End plot_data_two_iso function
plot_data2 <- function(filename, plot_save_pdf, plot_save_png, mix, source, discr) {
  # check that discr rownames match source_names
  if(!identical(rownames(discr$mu), source$source_names)) {
    stop(paste("*** Error: Source names do not match in source and discr data files. Please check your source and discr data file row names.", sep=""))
  }
  if(!identical(rownames(discr$sig2), source$source_names)) {
    stop(paste("*** Error: Source names do not match in source and discr data files. Please check your source and discr data file row names.", sep=""))
  }
  if(mix$n.iso==1) {
    plot_data_one_iso(mix, source, discr, filename, plot_save_pdf, plot_save_png)
  } else {
    for(iso1 in 1:(mix$n.iso-1)) {
      for(iso2 in (iso1+1):mix$n.iso) {
        plot_data_two_iso2(c(iso1, iso2), mix, source, discr, filename, plot_save_pdf, plot_save_png)
      }
    }
  }
} # end plot_data function

set.seed(1234)

## model 1: null process x residual

mix.1 <- load_mix_data(filename = "Chap2_Consumer.csv",
              iso_names = c("d13C", "d15N"),
              factors = c(NULL),
              fac_random = c(NULL),
              fac_nested = c(NULL),
              cont_effects = c(NULL))

source.1 <- load_source_data(filename = "Chap2_Source.csv",
                     source_factors = NULL,
                     conc_dep = FALSE,
                     data_type = "mean",
                     mix.1)

discr.1 <- load_discr_data(filename = "Chap2_Discrimination.csv", mix.1)

p <- plot_data2(plot_save_pdf = F, filename = "iso.png", plot_save_png = T, mix = mix.1,
     source = source.1, discr = discr.1)