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Describing the Gut Microbiome of Two Freshwater Mussel Species within the Tennessee River Basin

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DESCRIBING THE GUT MICROBIOME OF TWO FRESHWATER MUSSEL SPECIES WITHIN THE TENNESSEE RIVER BASIN

By

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ABSTRACT

ELIZABETH ELOISE COTTEN: Description of the gut microbiome of two freshwater mussel species within the Tennessee River Basin (Under the direction of Colin Jackson)

Freshwater mussels are important bioindicators of aquatic environmental quality, yet little is known about the composition of their gut microbiome or how it varies between different locations and mussel species. In this study, two species of mussels were collected from three sites located in two rivers, the Duck River and the Paint Rock River of the Tennessee River Basin. The gut microbiome of each mussel was characterized by 16S rRNA gene sequencing. Gut microbial communities were primarily composed of members of the Planctomycetes, Cyanobacteria, and Proteobacteria, which along with six other phyla accounted for nearly 90% of the sequences. Dominant operational taxonomic units (OTUs) included representatives of the Fusobacteria, Firmicutes, Planctomycetes, Cyanobacteria, and Proteobacteria. Gut microbial communities primarily differed based on sample site and were also influenced by mussel species and river. With site as the most significant influence on the gut microbiome, changes to the local environmental conditions are likely to affect this aspect of mussel biology. Therefore, freshwater conservation efforts should consider both the gut microbial community and the mussels themselves.

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Introduction

Host-associated microbes play a central role in host biology, ecology, and evolution (Simon, et *al.*, 2019). These microbes are key inhabitants of macroorganisms (Simon, et *al.*, 2019). Therefore, understanding host-microbiota interactions is crucial to the study of biology. Research reveals that host phenotypes involve not only expression of the host genome but also the expression of associated microbial genomes (Simon, et *al.*, 2019). This idea involves one specific unit of selection in evolution, a holobiont. The term 'holobiont' was coined by Lynn Margulis in 1990 in reference to a unified entity of symbiont and host (O'Malley, 2017). A holobiont is defined as a host and its associated communities of microorganisms and the hologenome describes the sum of the host genome and associated microbial genomes (Simon et *al.*, 2019). A holobiont with its hologenome functions as a unique biological entity and as a level of selection in evolution, and idea that is called the hologenome concept of evolution (Rosenberg, et al., 2008; Rosenberg & Zilber-Rosenberg, 2016; Suárez, 2018). This concept is based on four specific generalizations: First, the concept rests on the idea that a symbiotic relationship with microorganisms exists with all animals and plants (Rosenberg, et al., 2008). Second, transmission of symbiotic microorganisms occurs between generations. Third, that a host's association with its symbiont affects the fitness of the holobiont within its environment (Rosenberg, et al., 2008). Lastly, the hologenome concept of evolution acknowledges variation of the hologenome as the result of change in either the genome of the host or of the microbiota. This theory suggests the importance of the holobiont concept in the research of symbiotic relationships that bacteria share with their hosts.

Freshwater mussels are bivalve mollusks that share a similar structure with marine bivalves (Haag, 2012). Freshwater mussels display incredible diversity with over 300 species of Unionidae present in North America (Mulvey et *al.*, 1997). Historically, Unionid mussels served primarily as a food source with their shells and pearls serving as a common material used in jewelry. Unionidae are considered ecosystem engineers for their ability to significantly alter benthic habitats (Black et *al.*, 2017). These mussels excrete unused nutrients as solutes, predominantly ammonium and phosphate, which serve to nourish primary producers in the water column (Zieritz et *al.*, 2019). Remaining organic material is deposited by the mussels into the sediment as feces and pseudofeces, rich in ammonium and organic carbon (Black et *al.*, 2017). The clarity of aquatic habitats can depend on the filter-feeding process of mussels as they remove organic and inorganic particles, such as phytoplankton, zooplankton, and bacteria, from the water column (Zieritz et *al.*, 2019). Increased mussel densities can result in higher water clarity and subsequently affect the growth and composition of plankton and macrophytes (Zieritz et *al.*, 2019).

A mussel consists of an outer shell that protects the organism from outside harm and supports the organism's otherwise shapeless body mass. This outer shell is composed of two valves, with a hinge ligament and a pair of adductor muscles that work together to connect the valves (Haag, 2012). Shells of North American mussel species tend to reflect an oval or elliptical shape with moderate asymmetry. Mussels feed through a fundamental process known as filter feeding, a process that involves oxygen uptake, waste excretion, and gamete dispersal in addition to food intake (Haag, 2012). Freshwater mussels are suitable for use as biological indicators (Grabarkiewicz & Davis, 2008). As long-lived, sedentary borrowers who consume food and

oxygen by the filter-feeding process, mussels qualify as bioindicators (Grabarkiewicz & Davis, 2008).

The life cycle of a mussel is a complex process that involves a series of steps (Wolf, 2010). Male mussels release their gametes, sperm, into the water column. Once in the water column, sperm can be siphoned into the gills of a female mussel. The sperm then fertilize the female eggs, which are held in the gills. Fertilization success can be influenced by spatial aggregation of freshwater mussels, and the unionid mussel *Elliptio complanata* shows complete fertilization failure at local densities below ten mussels per square meter and a 100% success rate of fertilization at local densities that exceeded 40 mussels per square meter (Downing et *al.*, 1993).

Females' gills become slightly inflated upon fertilization and the female mussel is considered pregnant (Wolf, 2010). Female gills provide oxygen to fertilized eggs, and the eggs develop within the gills until reaching the larval stage. Mussel larvae are referred to as glochidia. Packets of glochidia, called conglutinates, are released and can be a food source for fish and other aquatic organisms. A female mussel may spawn multiple times during one reproductive season (Borcherding, 1991). Glochidia attach to fish and the fish acts as a host to the glochidia while the larvae mature. The larvae live as parasites for several weeks feeding on the bodily fluids of the host fish. The juvenile mussel remains attached to the host while it grows, until it is of sufficient size to drop off into the substrate (Wolf, 2010).

A key element of the mussel life cycle is the likely lack of microbiome transmission from parent to offspring, i.e. vertical transmission of the microbiome (Roughgarden, 2020). Vertical transmission incorporates microbes into a host lineage along with the host's nuclear genome, and inheritance of genes occurs through lineal descent (Roughgarden, 2020). In contrast, in

horizontal transmission of the microbiome, beneficial microbes within hosts are acquired from the environment, with other hosts potentially serving as sources of microbes such that inheritance of genes is accomplished by collective inheritance (Roughgarden, 2020). Such horizontal transmission of the microbiome is likely to contribute strongly to the gut bacterial composition in freshwater mussels.

Microbial communities such as the gut microbiome of freshwater mussels exhibit spatial variation at different scales as the microbiota share constant interaction with the host (Ruuskanen et *al.*, 2021). Spatial patterns were observed to have a positive correlation to microbial composition as sites close together have revealed more similar species composition than sites at further distance from each other (Briers & Biggs, 2005). Understanding what shapes the dynamics of microbiota under natural conditions is significant to the understanding of how organisms such as freshwater mussels may respond to environmental changes (Lokmer et *al.*, 2016). Analysis of spatial variation in previous studies revealed site and immediate environmental conditions are heavy influential on the host associated microbial communities (Lokmer et *al.*, 2016). The influence of spatial patterns on bacterial community composition can be as great as, or even greater than environmental factors (Lear et *al.* 2014).

This study examined the variation in the gut microbiome of two coexisting species of freshwater mussel, *Amblema plicata* and *Cyclonaias tuberculata*, Unionidae, found in the Duck River and the Paint Rock River of the Tennessee River Basin. The goal of this study was to assess whether species, site location, or river exert the strongest influence on the microbiome of *Amblema plicata* and *Cyclonaias tuberculata*. Based on feeding and reproductive behavior of Unionidae, I hypothesized that site would have the greatest effect on the gut microbial community of *Amblema plicata* and *Cyclonaias tuberculata* as there should be little vertical

transmission of the gut microbiome (i.e. reduced host species effect) and more dependence on bacterial acquired during the feeding process. I further expected that samples collected in closer proximity to one another from the same river would be more similar in bacterial composition. Alternative hypotheses would be that host species has a greater influence on bacterial composition, or that broader scale patterns (i.e. river system) would have a stronger effect on the gut microbial community than specific site.

Methods

Sample Collection

Samples were collected from two rivers in the Tennessee River Basin, the Paint Rock River, in northern Alabama, and the Duck River, in Tennessee. Sites were sampled as part of a larger study examining the gut microbiomes of freshwater mussels in the southeastern USA. A single site ("Columbia", 35.61,-81.02) was sampled on the Duck River on September 5th, 2019. Two sites ("Jones", 34.68,86.31, and "Butler's", 34.58,-86.30) were sampled on the Paint Rock River on July 23rd and July 24th, 2019, respectively. Mussels were flash frozen in liquid nitrogen to ensure a safe transportation to the University of Alabama. The samples remained frozen until processed for microbiome analysis approximately three months later. Samples were labeled by river, site, and species (Figure 1).

DNA Extraction and 16S rRNA Gene Sequencing

Prior to extraction of gut tissue, the samples were partially thawed. Gut tissue was cut from the dissected mussels using aseptic technique and placed into microcentrifuge tubes and refrozen prior to transport to the University of Mississippi. There, gut tissue was first ground into small pieces using a pellet pestle and then ground for a second time after the addition of solution DC1 from a Qiagen PowerSoil Pro Kits (Qiagen, Germantown, MD). DNA was extracted from ground tissue as described in the PowerSoil Pro kit protocol.

The V4 region of the 16S rRNA gene was amplified and sequenced. This amplification and sequencing followed the dual-index barcoded Illumina next-generation sequencing approach

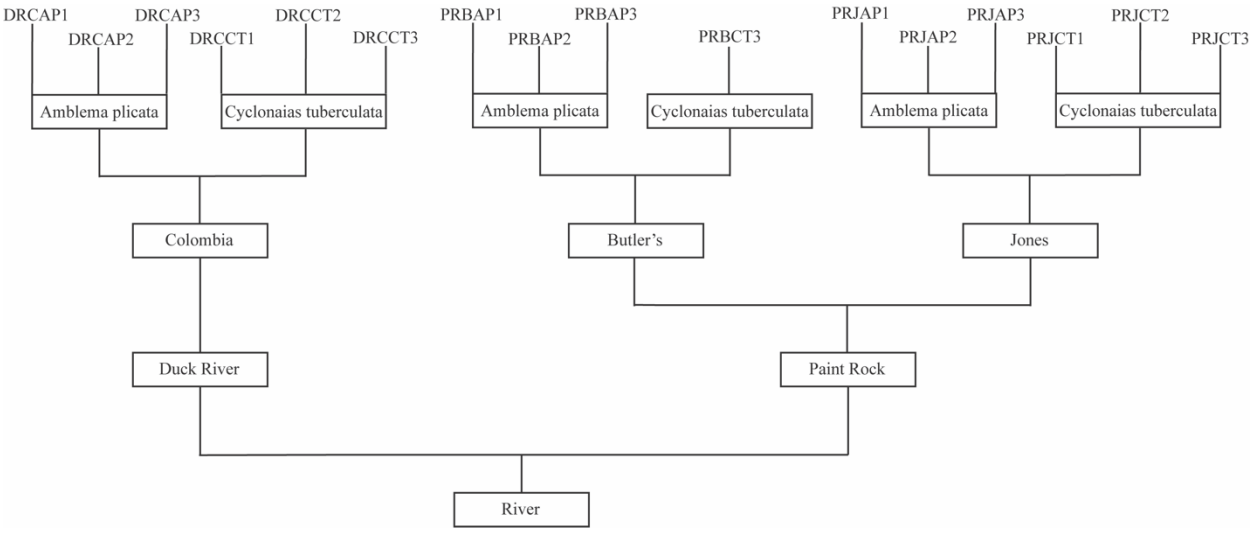

Figure 1) A breakdown of the ID names given to each sample. The first two letters of each name represent the river, DR for Duck River and PR for Paint Rock River. The third letter represents the site at that river the sample was collected, C for Columbia, B for Butler's, and J for Jones. The species identified is labeled with AP for *Amblema plicata* or CT for *Cyclonaias tuberculata.* Lastly, the number represents the replicate (1-3).

of Koziech et al. (2013) and Jackson et al. (2015). Sequencing was conducted using an Illumina MiSeq at the University of Mississippi Medical Center Molecular and Genomics Core Facility.

Data Analysis

Microbiome sequence data as FASTQ files and was analyzed using mothur (Schloss et al., 2009). As recommended by Schloss et al. (2011), a series of system commands were performed in order to remove ambiguous sequences and to align the sequences against the SILVA v4 16S rRNA database. Chimeras, a term used to describe erroneously combined sequences, were removed using UCHIME software. Contaminant sequences (those classified as Eukarya, Archaea, chloroplast, and mitochondrial sequences) were also removed from the dataset. The remaining aligned sequences were classified following the Ribosomal Database Project database (02.2016), and the bacterial sequences were arranged into operational taxonomic units (OTUs) based on >97% similarity in sequences. Communities were described based on the composition of bacterial taxa and compared based on the presence or absence of OTUs and the relative abundance of each OTU.

Results

Two *Cyclonaias tuberculata* samples from the Butler's site were removed from the dataset because both had less than 1,000 valid sequence reads. The final number of bacterial sequences across the 16 remaining samples totaled 118,310. The number of sequences obtained from each sample was highly variable (Figure 2). An *Amblema plicata* sample (DRCAP2) at the Columbia site (Duck River) had the most sequences (38,495) while another sample of this species (DRCAP1) at this site gave the lowest sequence count (1,198). The mean number of sequences across all 16 samples was 7,394.

In terms of bacterial phylum composition (Figure 3), mussel samples were primarily composed of Planctomycetes, Cyanobacteria, and Proteobacteria. Nine phyla composed nearly 90% of the sequences: Planctomycetes (25.0%), Cyanobacteria (17.1%), Proteobacteria (16.7%), Firmicutes (13.0%), Fusobacteria (6.8%), Actinobacteria (3.9%), Verrumcomicrobia (2.7%), Bacteroidetes (1.3%), and Acidobacteria (1.1%) (Figure 3). There were broad differences between the two rivers, with the Duck River samples being primarily composed of Cyanobacteria (34.3%) and Planctomycetes (28.7%), while the Paint Rock River samples were primarily composed of Proteobacteria (23.2%) and Planctomycetes (22.8%) (Figure 3) and included various phyla each represented by few sequences.

In total, 85.5% of sequences were classified to the class level (Figure 4). Nine bacterial classes composed over 90% of all sequences and include Planctomycetia (37.4%), Fusobacteriia (8.5%), Cyanobacteria (10.2%), Alphaproteobacteria (8.3%), Gammaproteobacteria (8.8%),

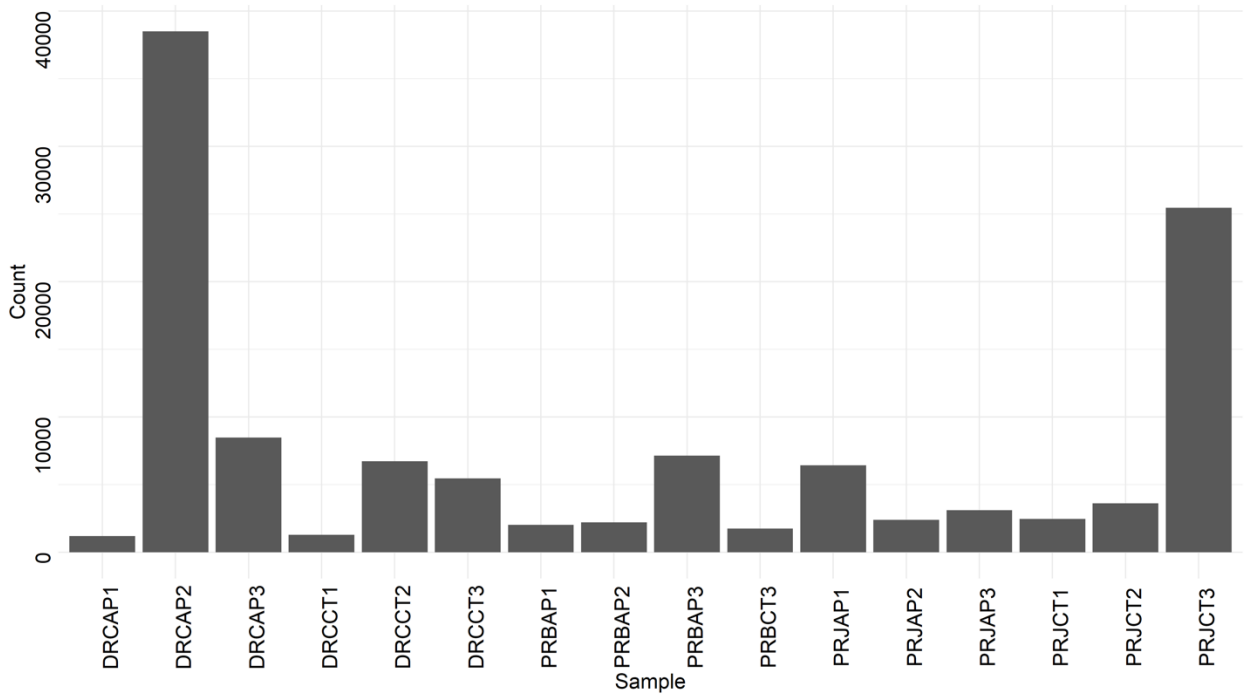

Figure 2) Numbers of 16S rRNA gene sequences obtained from next generation sequencing of bacterial communities of two freshwater mussel species, *Amblema plicata* and *Cyclonaias tuberculata* in two rivers in the Tennessee River Basin. Samples were taken from three sites on two rivers, the Duck River and the Paint Rock River. Sample ID is explained in Figure 1.

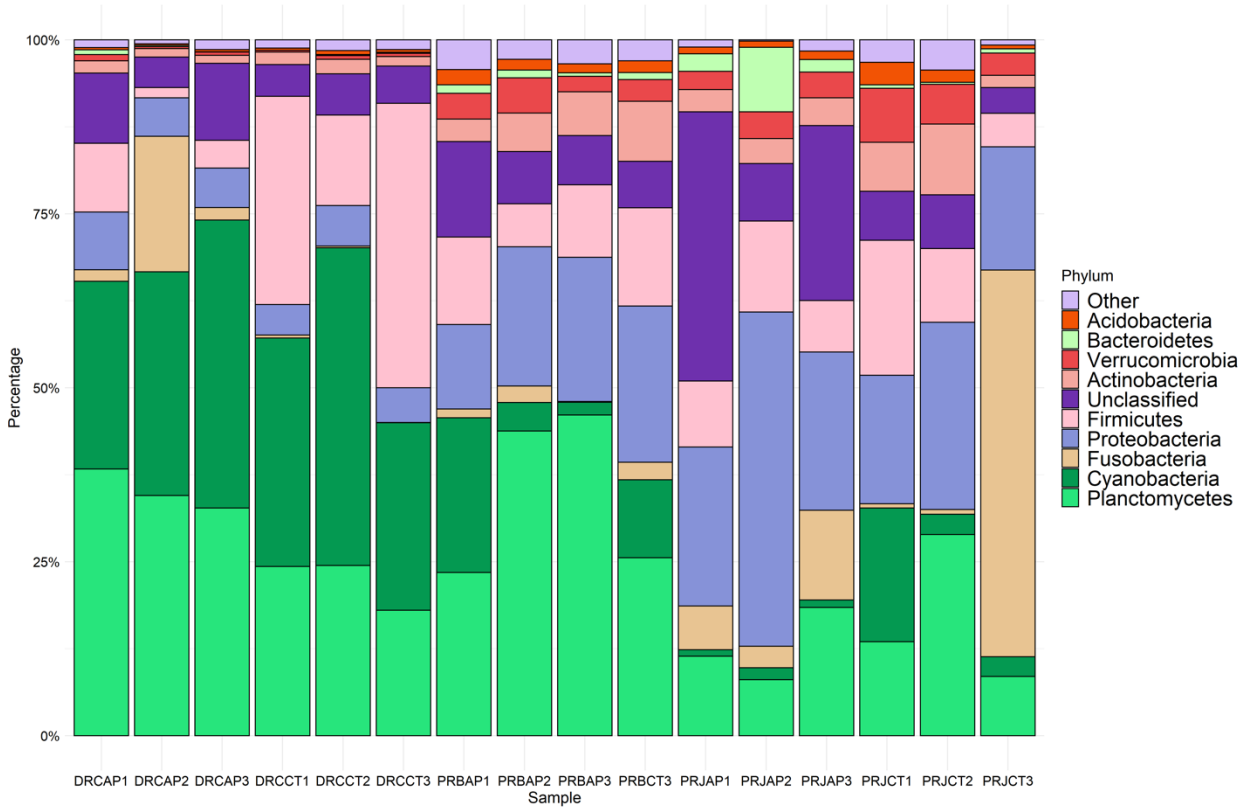

Figure 3) Relative abundance of bacterial phyla present in *Amblema plicata* and *Cyclonaias tuberculata* sampled from the Duck River and Paint Rock River. Chart includes sequences from 16 samples. The ten most abundant bacterial phyla are listed, while "Other" includes 14 additional phyla of bacteria. Sample ID is explained in Figure 1.

Betaproteobacteria (4.7%), Actinobacteria (5.5%), Clostridia (6.0%), and Bacilli (3.3%) (Figure 4). The most abundant classes of bacteria present included Planctomycetia and Cyanobacteria (Figure 4). Other abundant classes of bacteria included Gammaproteobacteria, Fusobacteriia, and Alphaproteobacteria (Figure 4). Gammaproteobacteria (12.8%) were abundant in all samples collected from the Paint Rock River (Figure 4).

To further understand the significance of the site location on the gut microbial community, relative abundance of bacterial composition at each site was determined. In terms of bacterial phylum abundance at each site, Columbia had a higher relative abundance of Cyanobacteria in comparison to Butler's (t-Test, $p<0.005$) and Jones (t-Test, $p<0.001$). Columbia had a significantly lower proportion of Proteobacteria in comparison to Butler's (t-Test, $p<0.001$) and Jones (t-Test, $p<0.001$). In terms of bacterial class abundance at each site, Columbia had a much greater percentage of Cyanobacteria than Butler's (t-Test, $p<0.001$) and Jones (t-Test, $p<0.001$). Columbia had a significantly lower relative abundance of Gammaproteobacteria in comparison to Butler's (t-Test, $p<0.001$) and Jones (t-Test, $p<0.001$).

Sequences were grouped into 4,474 OTUs based on 97% sequence similarity. 14 OTUs had >1000 reads. The most common OTU, identified as a member of Fusobacteria, accounted for greater than 20% of all sequences (Table 1). The second most common OTU, identified as a member of Planctomycetaceae, accounted for 18.3% of all sequences (Table 1). Two different OTUs, both identified as members of phylum Cyanobacteria, accounted for a combined total of an additional 12.1% of all sequences (Table 1). The remaining dominant OTU belonged to phylum Firmicutes and contained 5,464 reads with a relative abundance of 4.6% (Table 1). Two OTUs identified with a family taxonomic classification, OTU7 (1.8% of sequences) a member of

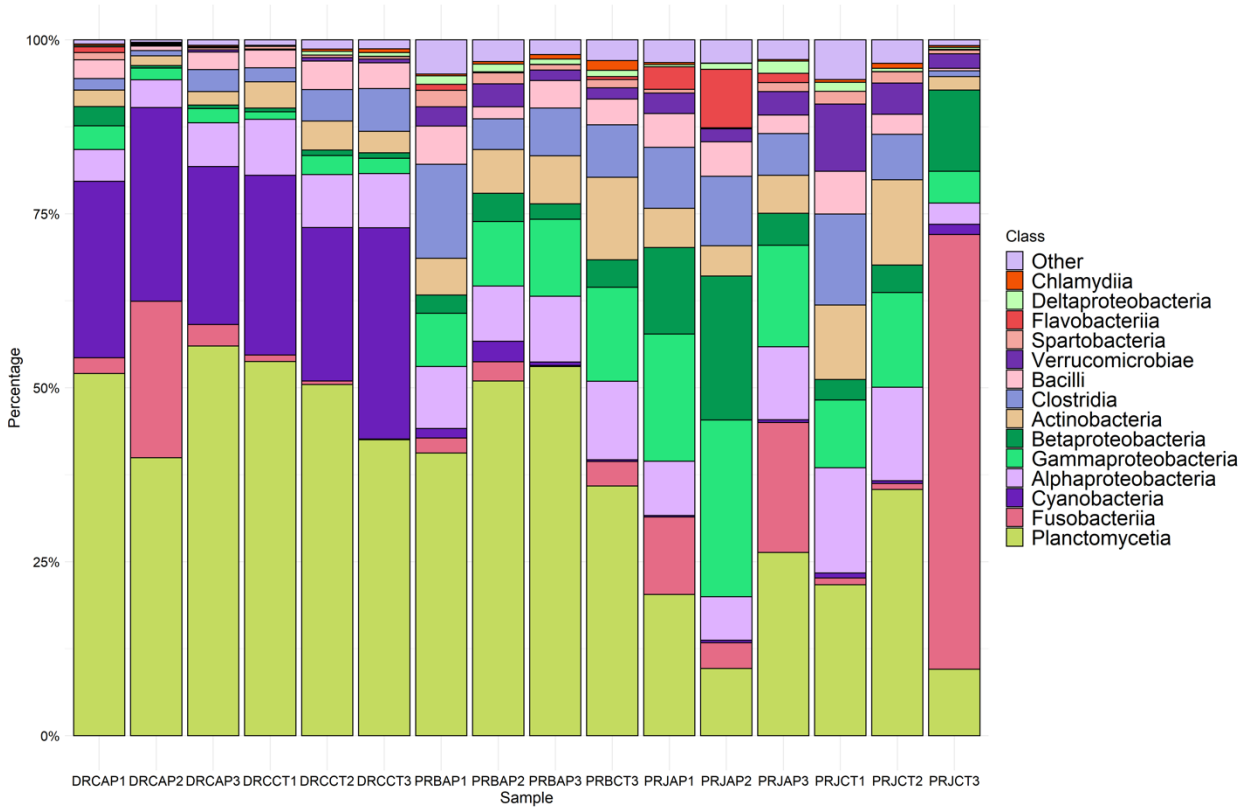

Figure 4) Relative abundance of bacterial classes present in *Amblema plicata* and *Cyclonaias tuberculata* sampled from the Duck River and Paint Rock River. Chart includes sequences from 16 samples. The 14 most abundant classes of bacteria are listed, while "Other" includes 11 additional different bacterial classes. A total of 69.85% of sequences were classified to the class level. Sample ID is explained in Figure 1.

the Methylocystis family and OTU10 (1.5% of sequences) a member of the Rubinisphera family were also relatively dominant (Table 1). Three OTUs presented unclassified bacteria and accounted for a combined total of 3.3% of all sequences (Table 1).

Neither site ($p>0.05$) nor species ($p>0.05$) were significant in OTU distribution. River location was significant in specific OTUs. OTU3, identified as Cyanobacteria, was significantly more present ($p<0.05$) in the Duck River samples when compared to the Paint Rock River samples. The same finding applied to OTU6 and OTU10, both identified as Planctomycetes, with p-values of <0.02 and <0.03, respectively.

The number of sequences in the sample (DRCAP1) with the fewest sequence reads (1,198) was used to determine the alpha diversity. Each sample was subsampled to 1,198 sequences and diversity indices determined. The sample with the highest observed species richness was a *Cyclonaias tuberculata* sample from the Jones site (Figure 5A). Additionally, two *Amblema plicata* samples and one *Cyclonaias tuberculata* sample from Butler's site, and one *Cyclonaias tuberculata* sample from Jones site exhibited similar species richness (Fig 5A). The samples with the lowest observed species richness include one *Amblema plicata* sample and an *Cyclonaias tuberculata* sample both collected from the Columbia site of the Duck River. The six samples with the highest species richness were all collected from the Paint Rock River.

The Inverse Simpson index involves both the number of species present and the relative abundance of each species. The highest Inverse Simpson value was represented by a *Cyclonaias tuberculata* sample collected from the Paint Rock River at the Jones site (Figure 5B). The *Amblema plicata* sample collected from the Paint Rock River at the Butler's site has the second highest Inverse Simpson (Figure 5B). All of the Duck River samples had low Inverse Simpson

Table 1) Most abundant bacterial OTUs found in samples of freshwater mussels taken from the Duck River and Paint Rock River. Only the OTUs with >1000 sequences are shown. Relative abundance is shown as a percentage of the total number of sequences in the dataset. OTUs are identified by phylum and the lowest taxonomic classification level of each that was possible. (c=class, d=domain, f=family).

OTU	Relative Abundance (%)	Phylum	Lowest Taxonomic Classification
Otu0001	20.32	Fusobacteria	(o)Fusobacteria
Otu0002	18.26	Planctomycetes	(o)Planctomycetaceae
Otu0003	7.52	Cyanobacteria	(p)Cyanobacteria
Otu0004	4.62	Firmicutes	(k)Firmicutes
Otu0005	4.61	Cyanobacteria	(p)Cyanobacteria
Otu0006	2.49	Planctomycetes	(o Planctomycetaceae
Otu0007	1.82	Proteobacteria	(f)Methylocystis

Otu0008	1.78	Planctomycetes	(o)Planctomycetaceae
Otu0009	1.68	Proteobacteria	(p)Betaproteobacteria
Otu0010	1.46	Planctomycetes	(f)Rubinisphaera
Otu0011	1.29	Bacteria_unclassified	(d)Bacteria
Otu0012	1.17	Proteobacteria	(c)Rhizobiales
Otu00013	1.05	Bacteria_unclassified	(d)Bacteria
Otu00014	0.92	Bacteria_unclassified	(d)Bacteria

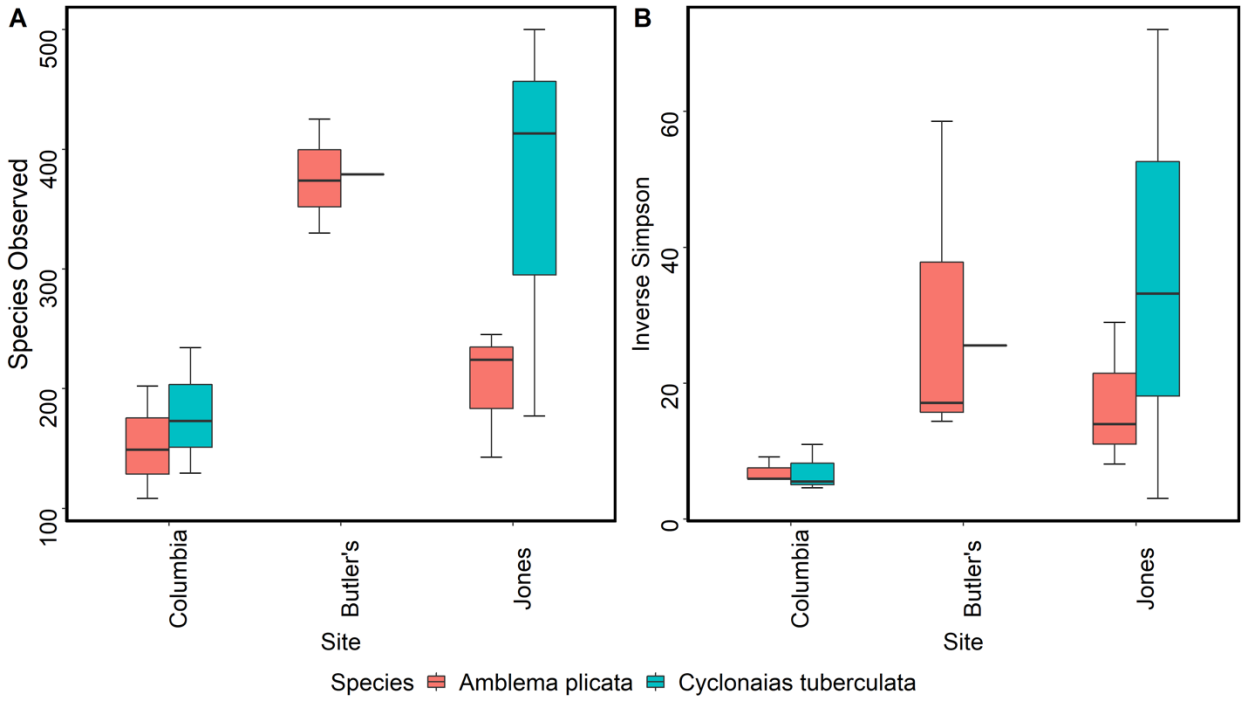

Figure 5) Diversity of bacterial communities in guts of *Amblema plicata* and *Cyclonaias tuberculata* sampled from the Duck River and Paint Rock River. Diversity was determined by subsampling of 1,198 sequences and expressed as (A) Species Observed (sobs), and (B) Inverse Simpson index. Figure 5A shows species observed. The dark line measures the number of species one would expect to see if 1,198 sequences were removed from each sample and the number of species was measured. The top and bottom lines show, statistically, the high and low confidence levels, or the highest and lowest number of species observed that may be present in each sample. Figure 5B shows Inverse Simpson Index. As with Figure A, the standard error bar is shown above and below each box. The top and bottom outline of each box show high and low confidence levels.

values. Two samples from the Paint Rock River at the Jones site, one *Amblema plicata* and one *Cyclonaias tuberculata*, shared Inverse Simpson values similar to the Duck River samples. Species evenness at each of these sites was low compared to other samples. The remaining Paint Rock River samples have a higher Inverse Simpson index in comparison to all Duck River samples and the two previously specified Paint Rock River samples.

A Bray-Curtis measure of dissimilarity, visualized using non-metric multidimensional scaling (NMDS, Figure 6), showed patterns in mussel gut microbiome composition and how it related to species, site location, and river. Multivariate analysis of variance (MANOVA) was used to test which of the three factors had the most effect on gut bacterial composition of *Amblema plicata* and *Cyclonaias tuberculata*. Site location was determined to be most significant in microbiome composition (MANOVA, $p<0.05$) and the strongest influence on the separation of samples on the NMDS plot. Pairwise comparison showed only Columbia and Jones were significantly different (MANOVA, $p= 0.0027$). These sites were also the farthest away on the NMDS plot. River was also significant (MANOVA, $p= 0.001$), but species was not (MANOVA, $p= 0.153$). All six Duck River-Columbia samples clustered together and three of the four Paint Rock River-Butler's site samples clustered close together. The Paint Rock River-Jones samples did not reveal the same clustering tendency evident in the Duck River-Columbia and Paint Rock River-Butler's sites (Figure 6).

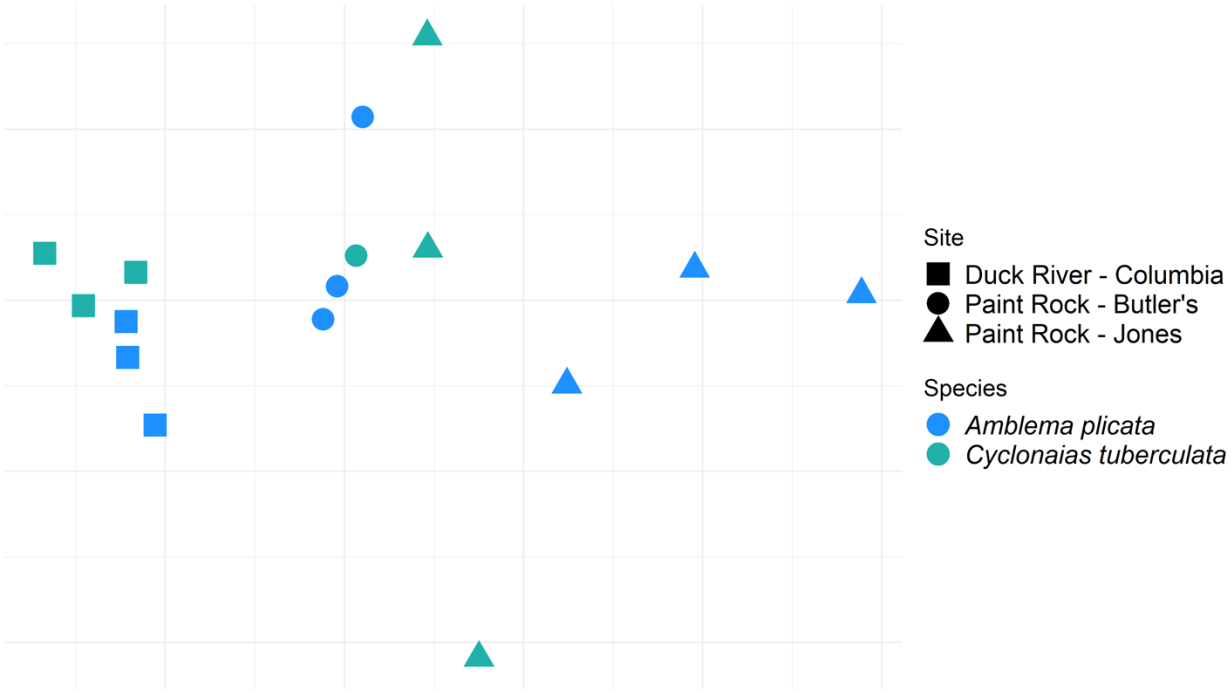

Figure 6) NMDS ordination (stress = 0.27) based on Bray Curtis dissimilarity showing patterns in gut microbiome composition of the freshwater mussels *Amblema plicata* and *Cyclonaias tuberculata* collected from the Duck River and the Paint Rock River. This Bray Curtis dissimilarity is based upon 118,310 total sequences. The minimum sequence count was 1198 sequences. There was a median of 7394 sequences present in each sample, and the max number of sequences found in one sample totaled to 38495 counts.

Discussion

This study used 16S rRNA gene sequencing to describe gut bacterial communities in two species of freshwater mussels, *Amblema plicata* and *Cyclonaias tuberculata*, in two rivers in the Tennessee River Basin. The most complex and abundantly populated microbial communities can be found in the gastrointestinal tract and comprise nearly 99% of the microbial biomass colonized within a host organism (Allen-Verceo *et al.* 2014). This gut microbial community is an intrinsic part of host physiology, and, therefore, research of the gut microbiome is essential. This study focused on the effect of host location on gut microbiome, and my findings suggest that site has the greatest effect on microbial diversity when compared to river and species.

Identification of a range of bacterial phyla and classes revealed the biodiversity present in the mussel gut microbial community. Planctomycetes, the most commonly detected phylum of bacteria present among the mussel samples, are widely distributed in aquatic environments, including freshwater with diverse environmental and physicochemical conditions (Kaboré et *al.*, 2020). Some members of the Planctomycetes play an important role in the global nitrogen cycle as some of them oxidize ammonium to dinitrogen (Fuerst et. al, 2011). Plantomycetes also accounted for a large percentage of the mussel microbiome in a study that investigated the effects of species or the environment on gut microbiome composition (Weingarten et *al.*, 2019), so appear to be common members of the mussel gut community.

Cyanobacteria, the second most detected bacterial phylum, was present in all samples. Cyanobacteria are photosynthetic prokaryotes known for their production of prolific secondary

metabolites (Mazard et. al, 2016). A high proportion of Cyanobacteria in the gut could imply a substantial proportion of the gut microbiome is transient and are absorbed from the water column. Research has determined that bivalves can have a strong effect on the nutrient dynamics in freshwater systems (Vaughn & Hakenkamp, 2008). This idea connects to my finding of site to have the biggest effect on gut microbiome and could further suggest that gut microbial communities are dominated by bacteria taken-up from that particular site. Weingarten et *al.* (2019) support the implication that bacterial retention within the gut is an active and potentially functionally selective process.

Proteobacteria and Firmicutes are two bacterial phyla detected in all samples. This finding is consistent with previous studies. Proteobacteria was the second most dominant phylum found in a study that analyzed the effects of propagation on microbial community structure in freshwater mussels (Aceves et *al.*, 2018). Firmicutes are commonly found at abundant levels of freshwater filter-feeding species, such as seen with *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis* fish (Liu *et al.* 2016). Weingarten et *al.* (2019) found Firmicutes to be the second most abundant phyla of bacteria in their examination of influence on microbiome composition of four co-occurring freshwater mussel species in the Sipsey River in Alabama, USA (Weingarten et *al.*, 2019). McCauley et *al.* (2021) examined temporal variation in the microbiome of six mussel species (none *Amblema plicata* or *Cyclonaias tuberculata*) sampled from the same river and found Proteobacteria and Firmicutes accounted for more than 50% of all sequences reads.

Fusobacteriia were among the most abundant classes of bacteria present in all samples. This finding is supported by other studies that analyze gut microbiota diversity of freshwater mussels, such as the study of Liu et al. (2020) that determined Fusobacteria to be the dominant

phylum in *Solenaia carinata* and *Sinohyriopsis cumingii* samples. While class Fusobacteriia has had little attention, the genus *Fusobacterium* includes obligately anaerobic, non-sporeforming, motile or nonmotile, Gram-negative rods that have been isolated from the human gastrointestinal tract (Hofstad, 1992).

Unclassified bacteria accounted for over 10% of bacterial phylum composition, and three common OTUs identified as members of unclassified bacteria. A similar study on the gut microbiome of freshwater Unionidae mussels found 16.2% of all sequence reads to be unclassified at the phylum level (Weingarten et *al.*, 2019). Results of most environmental microbiome studies show a high percentage of unclassified bacteria, because many bacteria remain yet to be discovered or cultured (Steen et *al.*, 2019). Inability to accurately and effectively recreate environmental conditions in culture limits experimental success in discovering new bacteria.

In regard to the aquatic environments of this study, the Tennessee River Basin is one of the most diverse temperate freshwater ecosystems in the world with more than 141 freshwater mussel species (Knight et *al.*, 2013). The Tennessee River Basin distributes its diverse aquatic fauna across 64,000km of water streams and rivers (Knight et *al.*, 2013). Site was found to be the most significant factor affecting gut microbiome composition, in comparison to species and river. Species was less influential on gut microbiome composition, although it does have some effect as clustering of samples in NMDS ordination is evident based on species. A prior study looking at the influence of species and site on four species of freshwater mussels contrasts this finding and found that the effect of species was more observable than the effect of site (Weingarten et *al.*, 2019). Samples taken from the Duck River at Columbia site and the Paint Rock River at Butler's site exhibit a tighter clustering by site than samples from the Paint Rock

River at the Jones site. This means that samples from the Jones site were more variable, perhaps because of more environmental variation at the Jones site.

The water filtration abilities of freshwater mussels can significantly benefit the surrounding environment. Without the presence of freshwater mussels, less algae and organic matter are removed from the water column, and light penetration to bottom-dwelling plants significantly decreases (Levy, 2019). Therefore, conservation efforts of freshwater mussel populations are crucial to maintaining viable environments for aquatic organisms and plants. Freshwater ecosystems are one of the most threatened habitats in the world as unprecedented pressures that result from human population and socioeconomic development cause habitat loss, habitat modification, climate change, and more (Lopes-Lima et *al.*, 2018). Specifically, the order Unionida is the most endangered group of freshwater bivalves (Lopes-Lima et *al.*, 2018). Freshwater mussel population sizes decline as large-scale impoundment of rivers has threatened mussel viability (Vaughn & Taylor, 2001). Effective conservation efforts require prioritizing scientific research and human actions to identify and mitigate risk factors to the health and viability of mussel species (Ferreira-Rodríguez et *al.*, 2019). One study compiled information on the occurrence and conservation status for freshwater mussel fauna of three states in the United States, including the species *Amblema plicata* that was sampled in this study (Carr & Francher, 2021). Many freshwater mussel species are declining in the project area, with poor water management and episodic events primarily being responsible (Carr & Francher, 2021). Management of downstream water levels and low flow rates during peak reservoir discharges is proven to promote population growth, as seen in populations of *Fusconaia flava* and *Lampsilis teres* between 1991 and 2003 (Carr & Francher, 2021). To maintain diverse abundance of aquatic fauna, the presence of freshwater mussels is essential. Freshwater mussels are crucial to

maintaining aquatic environments as their filter-feeding and excretion abilities impact the composition of the water column and the viability of the organisms that live within it. By investigating the gut microbial communities of freshwater mussels, we can approach future research of Unionidae with a better understanding of what factors are most significant in determining the gut microbiota of freshwater mussels.

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