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Description and characterization of the digestive gland microbiome in the freshwater mussel *Villosa nebulosa* (Bivalvia: Unionidae)

Alison K. Aceves[1], Paul Johnson[2], Stephen A. Bullard[3], Stacey Lafrentz[1] and Cova R. Arias[1]

[1]*Aquatic Microbiology Laboratory, School of Fisheries, Aquaculture, and Aquatic Sciences, 559 Devall Drive, Auburn, AL 36849, USA;*
[2]*Aquatic Parasitology Laboratory, School of Fisheries, Aquaculture, and Aquatic Sciences, 203 Swingle Hall, Auburn, AL 26849, USA; and*
[3]*Alabama Aquatic Biodiversity Center, 2200 Highway 175, Marion, AL 36756, USA*

Correspondence: C. R. Arias; e-mail: ariascr@auburn.edu

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ABSTRACT

Here we characterize the digestive gland microbiome from wild and cultured (hatchery-reared) Alabama rainbows (*Villosa nebulosa*) using 16 S rRNA gene pyrosequencing in order to understand the effects of propagation on microbial community structure in freshwater mussels. Digestive glands from nine Alabama rainbows were analysed, five from the wild and four from a hatchery. Pyrosequencing yielded a total of 32,962 bacterial sequences and 387 operational taxonomic units (OTUs). Tenericutes was the dominant phylum in all samples (>87%), followed by Proteobacteria (4.6%), Fusobacteria (4.5%) and Bacteroidetes (1.4%). Digestive gland microbiomes were overwhelmingly dominated by OTUs related to the genus *Mycoplasma*. These *Mycoplasma*-like sequences could not be ascribed unequivocally to the genus *Mycoplasma* (less than 90% sequence identity) and probably represent new lineages within the class Mollicutes. We identified a core microbiome in the digestive gland of *V. nebulosa*, with all individual mussels sharing 9 OTUs. However, the microbiome from mussels collected from the wild was significantly different from that of hatchery-reared mussels. Our results show that novel microbial communities exist within the digestive gland of freshwater mussels.

INTRODUCTION

Freshwater mussels (Unionoida: Unionidae) are filter-feeding parasitic bivalves that, following the fish-parasitic glochidial larval stage, reside in sediment and consume or otherwise process bacteria, phytoplankton, detritus and particulate organic matter from the water column (Silverman *et al.*, 1995; Vaughn & Hakenkamp, 2001). Freshwater mussels perform important ecosystem services, e.g. turning over sediments (Gutierrez *et al.*, 2003; Vaughan, 2017), filtering water and maintaining its quality (Naimo, 1995; Mcgregor & Garner, 2004; Atkinson *et al.*, 2013), and translocating nutrients from the water column to the benthos, thereby making resources available to organisms at other trophic levels (Vaughn & Hakenkamp, 2001; Howard & Cuffey, 2006; Atkinson *et al.*, 2013). Historically, freshwater mussels dominated the benthos of rivers and lakes in eastern North America (Strayer, 2008; Vaughan, 2017), but are now the most imperiled North American faunal group (Williams *et al.*, 1993; Bogan, 2008). Population declines and species extirpations have resulted from physical modification of riverine habitats, water quality degradation and introduction of invasive species (Williams *et al.*, 1993; Bogan, 2008).

Much of the literature on freshwater mussels has focused on feeding behaviour (Vaughan, 2017) and diet (Silverman *et al.*, 1995; Vaughn & Hakenkamp, 2001; Christian *et al.*, 2004; Atkinson *et al.*, 2013); however, the role of gut-bound bacterial symbionts is underexplored. This represents a significant gap in our knowledge of the biology of freshwater mussels, because such bacteria in other metazoan lineages are likely critical for nutrient assimilation (Mueller *et al.*, 2012). Studies of economically important molluscs such as abalone (*Haliotis diversicolor*) have suggested that microbial communities play an important role in algal polysaccharide degradation and maintenance of pH and redox potential in the gut (Tanaka *et al.*, 2003). This study is among the first to present evidence of microbial endosymbionts in freshwater mussels. It also considers environmental effects on microbial community structure, by contrasting the bacterial communities in conspecific hatchery-reared and wild-caught freshwater mussels.

The association between freshwater mussels and their gut microbes is generally attributed to the ingestion of bacteria (Harris, 1993). This host-microbe interaction may be the direct result of the bivalve consuming and digesting microbes (Silverman *et al.*, 1995; Nichols & Garling, 2000; Christian *et al.*, 2004) or alternatively the bacteria in the gut may be transient or commensal (Harris, 1993). To date, most studies evaluating the microbial communities of freshwater mussels have focused on identifying potential pathogens (Starliper *et al.*, 2008; Grizzle & Brunners, 2009). Starliper *et al.* (2008) investigated the normal microbiota of healthy freshwater mussels from the Holston and Clinch rivers in Virginia to identify potential bacterial pathogens. Similarly, Chittick *et al.* (2001) assessed the health status of *Elliptio complanata* from North Carolina by culturing the digestive gland as a means of assessing bacterial community diversity. Although these

Table 1. Mussel origin and diversity indexes as calculated by MOTHUR (v. 1.33.3) software.

Sample ID	Group	# Observed OTUs	Good's coverage	# Predicted OTUs		Shannon evenness
				ACE	Chao1	
DG4	Wild	71	0.955326	62.95034	45	0.710173
DG6	Wild	68	0.876289	158.5175	123.5	0.794941
DG7	Wild	39	0.893471	109.1578	101.2143	0.813071
DG8	Wild	44	0.941581	84.54034	52.6	0.736632
DG10	Wild	32	0.931271	66.46605	78	0.698693
DG78	Hatchery	40	0.931271	70.04549	73	0.757073
DG79	Hatchery	54	0.920962	152.0461	72.11111	0.739279
DG80	Hatchery	49	0.920962	113.382	68.46154	0.69695
DG81	Hatchery	49	0.931271	75.29514	62.57143	0.742022

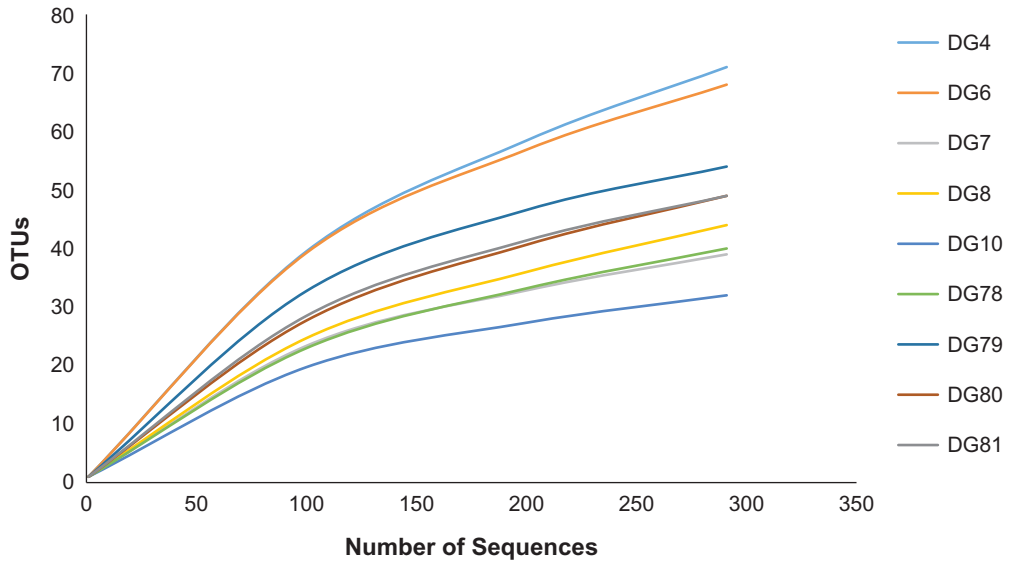

Figure 1. Rarefaction curves of individual *Villosa nebulosa* analysed in the study. Sequences were standardized to equal sample sizes for direct comparison. Wild mussels: DG4-10; hatchery-reared mussels: DG78-81.

studies provided important baseline data for mussel health, they all employed culture-based methods, which routinely recover less than one tenth of the total microbial diversity (Amann, Ludwig & Schleifer, 1995). Recently, molecular-based techniques have been developed in several marine bivalve species, which characterize the microbial communities without the need to isolate and culture specific microbes. Such molecular studies have characterized the microbiome in the gill, stomach, gut and whole homogenate of oysters (Romero *et al.*, 2002; Hernandez-Zarate & Olmos-Soto, 2006; King *et al.*, 2012), estuarine mussels (*Brachidontes* sp.) from Indonesia (Cleary *et al.*, 2015), abalone (Huang *et al.*, 2010) and the freshwater zebra mussel (*Dreissena polymorpha*) (Winters, Marsh & Faisal, 2011). Because of their value as seafood (Keithly & Diop, 2011) and because they carry human-pathogenic bacteria (Kelly & Dinuzzo, 1985), much effort has been placed on characterizing the microbiota of oysters (Trabal *et al.*, 2012; Lokmer & Wegner, 2015).

A recent review (Bahrndorff *et al.*, 2016) suggested that the microbiome could have significant implications for conservation biology, especially for threatened or endangered species. Freshwater mussels are recognized as an important component of river ecosystems (Vaughan, 2017) and recovery efforts through propagation and reintroduction are in progress across several southeastern states of the USA (Barnhart, 2006a). One conservation target is the Alabama rainbow, *Villosa nebulosa* (Conrad, 1834), which has been petitioned for federal protection under the Endangered Species Act. Research and recovery efforts for *V. nebulosa* are led by the Alabama Aquatic Biodiversity Center (AABC) and reintroductions began several years ago. The species is a Mobile River Basin endemic and its historic distribution included the upper Coosa, Cahaba and Warrior river basins above the Fall Line (Williams *et al.*, 1993).

With the aim of assisting ongoing recovery efforts, we here characterize and contrast the microbiome of the digestive gland from cultured and in-stream *V. nebulosa*, using 16 S rRNA gene pyrosequencing. No previous study has determined the bacterial composition and diversity of the digestive gland of any freshwater mussel using genomic methods. We hypothesize that significant differences exist between the gut microbiomes of cultured *versus* in-stream mussels.

MATERIAL AND METHODS

Sample collection

Five mature female *Villosa nebulosa* were collected from Terrapin Creek, Cleburne Co., Alabama (33.861306°N, 85.5225730°W) on 1 May 2011 and transported to Auburn University for analyses ('wild mussels': DG4, DG6, DG7, DG8 and DG10). The cultured

V. nebulosa were produced by transforming glochidia that infected the gill of Coosa bass (*Micropterus coosae*) and rearing the juveniles in upwelling chambers (Barnhart, 2006b). Juvenile *V. nebulosa* were fed a mix of commercially available *Nanochloropsis* species and shellfish diet (Reed Mariculture) added to hatchery pond surface water filtered to 120 µm. After 60–90 d post-transformation, juveniles were transferred to suspended upwelling systems (SUPSYS) deployed in an AABC rearing pond, where they were kept for *c.*15 months ('hatchery mussels': DG78, DG79, DG80 and DG81) before being shipped alive to Auburn University.

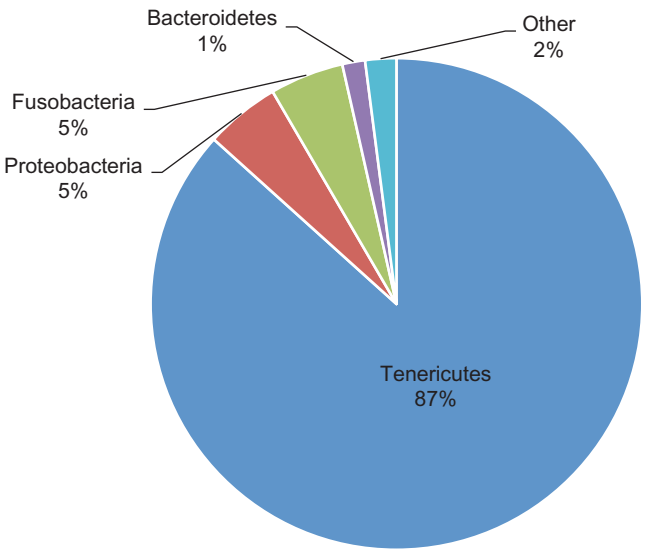

Figure 2. Phylum composition of the digestive gland microbiome of *Villosa nebulosa*.

DNA extraction

Approximately 25 mg of digestive gland tissue was aseptically collected from each mussel. DNA extraction was carried out using DNeasy Blood & Tissue Kit (Qiagen, CA) following Gram-positive bacterial DNA extraction. Extracted DNA was quantified by photometry using a Nanodrop 2000 (Thermo Scientific, Rochester, NY) and the quantities adjusted to 20 ng/µl. PCR suitability of samples was confirmed by using universal primers for the 16 S rRNA gene (Larsen *et al.*, 2015) and obtaining a clear amplicon of the expected size. Samples were kept at –20 °C until sequencing. Roche titanium 454 sequencing was performed on 10 digestive gland samples (five in-stream, four hatchery-reared) using individual barcodes and primer 27 F (5′-AGRGTTT-GATCMTGGCTCAG-3′) amplifying the variable V1–V3 region of the 16 S rRNA. PCR conditions were as follows: initial denaturation at 94 °C for 3 min and 30 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, including a final elongation at 72 °C for 5 min. The resulting sequences were processed with an exclusive analysis pipeline (MR DNA, Shallowater, TX) including removal of barcodes and primers as well as sequences of less than 200 bp, a base call error rate of less than 0.3% ($Q < 25$), ambiguous base calls and long (>6 bp) stretches of identical bases. Following removal of noise, and of chimera and singleton sequences, operational taxonomic units (OTUs) were defined in agreement with the accepted prokaryotic species concept (>3% sequence agreement; Rossello-Mora & Amann, 2001) and identified taxonomically using BLASTn against the Greengenes database (Desantis *et al.*, 2006).

Data analysis

The mussel with the fewest number of total sequences ($n = 291$) was used for standardization for diversity analyses and rarefaction curves for the microbial communities identified from all other mussels. Using Mothur v. 1.33.3 software (Schloss *et al.*, 2009), rarefaction curves, Good's coverage, abundance-based coverage

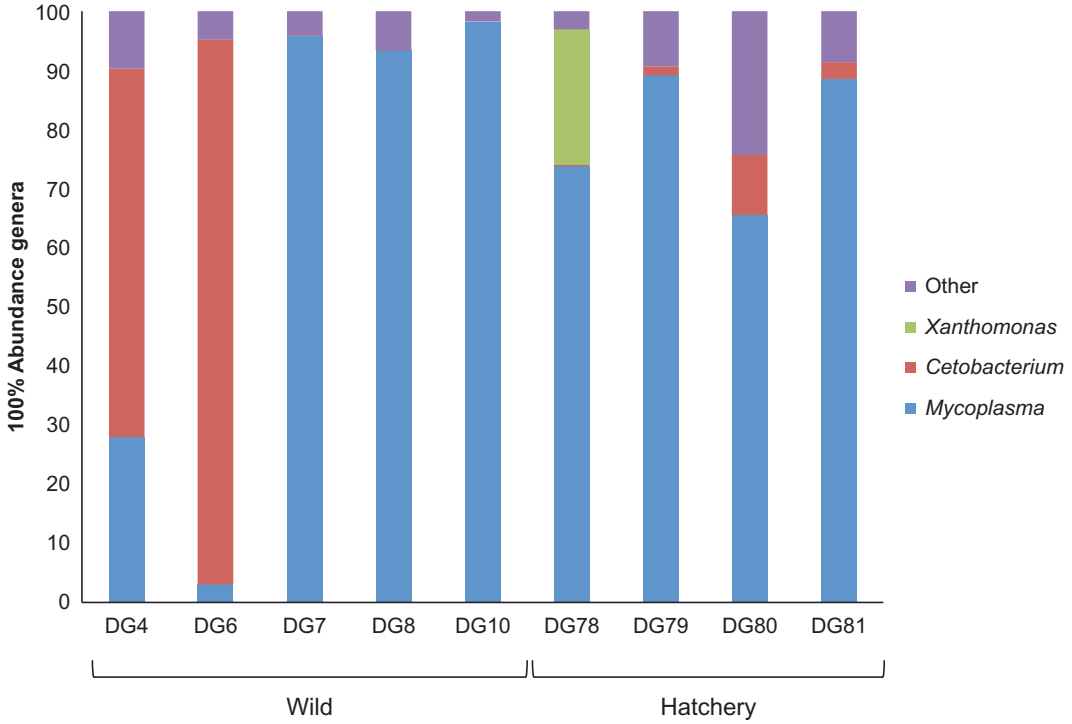

Figure 3. Distribution of predominant genera in digestive-gland microbiome of each individual *Villosa nebulosa*.

Table 2. Percent abundance of bacterial genera found in digestive glands of *Villosa nebulosa* (only top five genera from each individual mussel are listed).

Mussel	Bacterial genus	Percentage abundance
DG4–Wild	*Cetobacterium*	62.28
	Mycoplasma-like	27.98
	Lactobacillus	3.61
	Ralstonia	1.10
	Dysgonomona	1.00
DG6–Wild	*Cetobacterium*	92.10
	Aeromonas	3.44
	Mycoplasma-like	3.10
	Shewanella	0.69
	Klebsiellan	0.34
	Parabacteroides	0.34
	Cetobacterium	92.10
DG7–Wild	*Mycoplasma*-like	95.82
	Acidovorax	1.94
	Acinetobacter	1.08
	Chryseobacterium	0.26
	Hyphomicrobium	0.10
DG8–Wild	*Mycoplasma*-like	93.36
	Acinetobacter	0.84
	Prevotella	0.81
	Acidovorax	0.66
	Akkermansia	0.47
DG10–Wild	*Mycoplasma*-like	98.26
	Acidovorax	0.51
	Acinetobacter	0.51
	Spiroplasma	0.28
	Edaphobacter	0.11
	Pseudomonas	0.11
DG78–Hatchery	*Mycoplasma*-like	73.74
	Xanthomonas	22.84
	Flavobacterium	0.69
	NC10 (Candidate division)	0.46
	Ureaplasma	0.42
DG79–Hatchery	*Mycoplasma*-like	89.51
	Cetobacterium	1.53
	Flavobacterium	1.31
	Microbacterium	0.98
	Sphingomonas	0.95
DG80–Hatchery	*Mycoplasma*-like	65.53
	Cetobacterium	10.11
	Flavobacterium	5.19
	Acinetobacter	4.87
	Pseudomonas	3.73
DG81–Hatchery	*Mycoplasma*-like	88.53
	Cetobacterium	2.80
	Staphylococcus	1.44
	Fusobacterium	1.41
	Flavobacterium	0.74

estimation (ACE), Chao1, Shannon evenness, observed OTUs and shared OTUs were generated. A one-way ANOVA was performed on all diversity indices. An OTU abundance table was loaded into PRIMER v. 6 (Clarke & Warwick, 2001) to perform similarity percentages (SIMPER) analysis in order to determine OTU differences between the individual microbial communities. The cut-off for low contributions was set at the default of 90.

RESULTS

Diversity analysis

Pyrosequencing of the 16 S rRNA gene yielded a total of 32,962 bacterial sequences and 387 OTUs. After standardization, 291 sequences and 247 OTUs remained. Sequence coverage was ≥89% for all sampled individuals of *Villosa nebulosa* (Table 1). Total expected richness was calculated by ACE and Chao1, but no significant difference between wild *versus* hatchery mussels was found. Individual rarefaction curves displaying the sequence coverage for each digestive gland sample are shown in Figure 1.

Digestive gland microbiome composition

Collectively, 16 bacterial phyla were identified from the digestive glands of the sampled Alabama rainbows (Fig. 2) although only four phyla represented more than 1% of all OTUs. The phylum Tenericutes dominated all samples analysed, represented by >87% of OTUs. OTUs of Proteobacteria were the second most common (4.6%), followed by Fusobacteria (4.5%) and Bacteroidetes (1.4%). Within the Proteobacteria, each mussel microbiome contained OTUs assigned to Gammaproteobacteria (4.5%) and Betaproteobacteria (2.9%). Less common phyla varied in abundance between mussels, e.g. DG10 was unique in having no representation of Fusobacteria, while DG6 lacked any Bacteroides (data not shown).

At the genus level, microbial diversity of *V. nebulosa* digestive gland microbiomes was dominated by OTUs that were similar to sequences of *Mycoplasma* species deposited in GenBank and GreenGenes; however, the microbial OTUs from the sampled mussels were largely unique, i.e. percentage identities between our samples and microbial OTUs in GenBank and GreenGenes was low (74–92%). On average, these OTUs only shared 81% sequence identity with known sequences from *Mycoplasma* species and we therefore refer to them as '*Mycoplasma*-like;' they likely represent a species or group of species that should be assigned to a new genus. We detected OTUs of the *Mycoplasma*-like clade in all digestive glands sequenced (Fig. 3). *Cetobacterium* dominated the microbiome in DG4 and DG6 only; however, OTUs of the *Mycoplasma*-like clade also were present. These two individuals had nodular masses on their mantle and appeared emaciated, indicative of poor health. *Cetobacterium* (the second most abundant genus in our samples) was present in 6 of the 9 mussels. Other genera (Table 2) were present in only single mussels and typically at low percentages. The genus *Xanthomonas* comprised >22% of the community in DG78, but was absent from all other samples.

A multidimensional-scaling (MDS) plot based on digestive gland OTU abundances was generated in PRIMER v. 6 in order to visualize clustering patterns related to the origin of the mussels sampled (Fig. 4). The MDS plot showed that bacterial composition was influenced by origin, with OTUs from hatchery-reared mussels forming a tighter cluster than those collected from the wild. The clusters were supported by ANOSIM with a global R value of 0.724 ($p = 0.04$) for origin.

A total of 9 OTUs were shared between the wild and hatchery-reared mussels, representing 4% of the total OTUs (Fig. 5). SIMPER analysis by OTUs revealed large differences in digestive gland bacterial communities between wild and hatchery-reared mussels. Among the wild mussels, the highest contribution of similarity was OTU-6 and among hatchery-reared musselss was OTU-47, both of which were *Mycoplasma*-like sequences. These

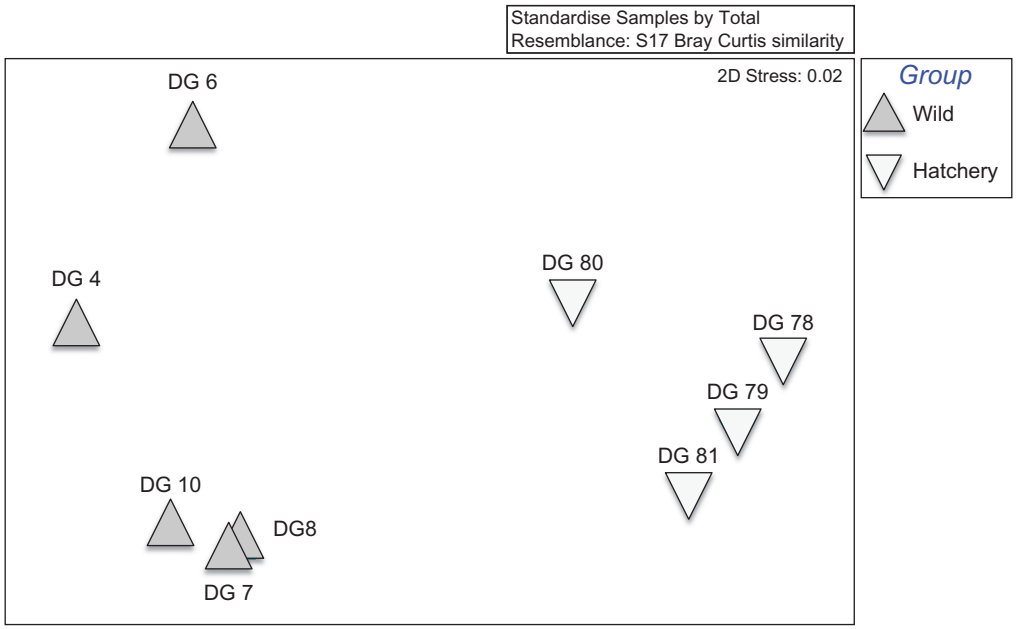

Figure 4. Multidimensional scaling of digestive gland samples according to mussel origin, based on percentage similarity in OTU abundances.

Figure 5. Venn diagram showing the number of shared and unique OTUs in microbial microbiome from digestive gland of wild and hatchery-reared *Villosa nebulosa*.

results indicate that OTU-47 makes the largest contribution to the dissimilarity between wild and hatchery mussels, followed by OTU-6.

DISCUSSION

The core microbiome of a species is defined as the group of microbes present in all individuals regardless of the environment (Turnbaugh *et al.*, 2007). Characterization of the core microbiome of freshwater mussels should facilitate culture efforts, not only to improve survivorship and production, but eventually to identify 'normal' or 'healthy' core microbial communities. However, understanding of the structure and diversity of core microbiomes across a number of mussel species will be required before evaluation of mussel health is possible. Core microbiome data could also facilitate evaluation of mussel mortality during kill events or disease epizooties (Southwick & Loftus, 2003). Several studies have attempted to characterize the core gut microbiomes of commercially important fish species (Tarnecki, Burgos & Arias, 2017), but few have focused on aquatic invertebrates. King *et al.* (2012) characterized the stomach and gut core microbiomes of the oyster *Crassostrea virginica* from two localities. The authors reported that core gut and stomach microbiomes were different, the core stomach microbiome having a lower alpha-diversity than that in the gut (the latter representing about 16% of all OTUs). The existence of a core microbiome in *C. virginica* was also supported by Pierce *et al.* (2015), in a study suggesting that seasonality had a stronger effect on the gut microbiome than locality. However, Trabal *et al.* (2012) reported geographic location as the primary driver of the microbiome in the oyster gut. In our study, we focused on a single unionid species, *Villosa nebulosa*, reared under two different conditions. Although we found significant differences in alpha diversity between wild and hatchery-reared mussels, all individual shared 11 of the OTUs, suggesting that a core microbiome exists in this species.

Our results showed no significant differences in terms diversity and evenness of OTUs between wild and hatchery-reared mussels, suggesting that these two groups had the same degree of bacterial diversity in their digestive glands, although species composition varied significantly. Overall, both groups were dominated by OTUs ascribed to the phylum Tenericutes, in particular to the class Mollicutes. Previous studies have identified Mollicutes as the dominant constituent of bacterial communities from a marine mussel, *Brachiodontes* sp. (Cleary *et al.*, 2015), from the oyster *Saccostrea glomerata* (Green & Barnes, 2010) and in the intestine of abalones, *Haliotis discus hannai* (Tanaka *et al.*, 2004). Even so, our findings were surprising because Mollicutes comprised as much as 98% of the total sequences identified in some of the *V. nebulosa*. Interestingly, our Mollicutes-OTUs had strikingly low similarity to previously sequenced microbial OTUs associated with marine molluscs. Kostanjsek, Strus & Avgustin (2007) reported a similar problem when they characterized the gut microbial community of the terrestrial isopod *Porcellio scaber*. After an extensive microscopic characterization of the bacteria associated with the hindgut wall of the isopod, they proposed '*Candidatus* Bacilloplasma' as a new lineage within Mollicutes to accommodate their newly-identified sequences and reported that the average similarity between new and previously sequenced microbes was below 82.6%. Similarly, our *Mycoplasma*-like OTUs share an average of 81% sequence similarity with those deposited in public databases. Our *Mycoplasma*-like OTUs could represent one or more novel lineages within the class Mollicutes; however, further phylogenetic studies and ultrastructure characterization of these putatively new bacteria are required before a new lineage is formally proposed.

The genus *Mycoplasma* consists of Gram-positive bacteria that are phylogenetically related to the *Bacillus/Clostridium* branch of the Firmicutes. Mycoplasmas lack a cell wall, have a low G + C content and have the smallest genome of any known self-replicating organism. Phylogenetic analyses indicate that mycoplasmas underwent multiple reductions in genome size (Joblin & Naylor, 2002). Because of their small genomes, they are unable to perform many basic metabolic functions and are considered obligate commensals or parasites (no free-living mycoplasmas have been identified to date). Mycoplasmas are typically associated with respiratory or urogenital mucosae, where they attach to the host eukaryotic cell through their tip organelle. In same cases, they become intracellular pathogens, but under appropriate environmental conditions most remain a benign member of the host's microbiome (Brown *et al.*, 2005). Some are associated with chronic illnesses in humans, whereas others are well-known pathogens, e.g. *Mycoplasma pneumonia* and *M. gallisepticum*. Because of the large number of *Mycoplasma*-like OTUs identified in our study, it is tempting to speculate that they confer some benefit to their host. Wang *et al.* (2016) assembled two draft genomes of mycoplasmas found in the stomach of the deep-sea isopod *Bathynomus giganteus* and performed a comparative genome analyses with four previously sequenced mycoplasma genomes (including *Candidatus* Hepatoplasma crinochetorum isolated from the terrestrial isopod *P. scaber*; Leclerq *et al.*, 2014), finding sialic acid lyase genes that can block attachment of pathogenic bacteria to the stomach wall, thereby protecting the host from invading pathogens. In addition, Wang *et al.* (2016) found multiple copies of genes related to proteolysis and oligosaccharide degradation and speculated that these genes may help the host survive under low-nutrient conditions.

This is the first study to evaluate the microbiome of a unionid species using next-generation sequencing. Our results revealed that the phylum Tenericutes, in particular the class Mollicutes, dominates the gut microbiome. The only exceptions were the two wild *V. nebulosa* that appeared emaciated, but further study is required to explore this aspect. Studies are ongoing to characterize further the mycoplasmas found in *V. nebulosa* and to explore the gut microbiome of other species of freshwater mussels in natural and hatchery settings. Our initial data indicate a much greater diversity of mycoplasma-like bacteria in the gut of this freshwater mussel than that reported from the gut of an isopod (Kostanjsek *et al.*, 2007). Further evaluation of this microbiome will require a much more powerful whole-genome sequencing approach.

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