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REGULAR ARTICLE

DIVERSITY AND PREDICTED FUNCTION OF GUT MICROBES FROM TWO SPECIES OF VIVIPARID SNAILS

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ABSTRACT

Animal gut bacteria are involved in numerous critical functions. In snails, gut bacteria play crucial roles in organic material digestion and nutrient production and have been implicated in aspects of reproduction. Snail gut microbes are known to differ between species and even between anatomical compartments of the digestive tract; dietary changes are also known to alter snail gut flora. In an effort to better understand their diversity and function, we studied the gut microbial communities from two viviparid snails, *Campeloma decisum* and *Cipangopaludina japonica*. We were interested in whether significant differences in bacterial community composition existed between the two species, and whether differences in microbial diversity corresponded to differences in community function. Using next-generation sequencing of the bacterial 16S V4 region, we found no significant differences in alpha and beta diversity between *Ca. decisum* and *Ci. japonica*. Firmicutes and Proteobacteria were the most abundant bacterial phyla in both species, while Bacteroidetes had a higher mean abundance in *Ci. japonica*. Nine taxonomic groups were present in significantly different mean abundances between the snail species. Pseudomonads and Enterobacteriaceae were notably more abundant in *Ca. decisum*, while Proteobacteria and Chitinophagaceae were more abundant in *Ci. japonica*. Peptidoglycan synthesis, pyruvate fermentation, and aerobic respiration by cytochrome *c* were the three most abundant microbial pathways represented in the viviparid gut. Fourteen functional pathways differed significantly between *Ca. decisum* and *Ci. japonica*, potentially correlated with differences in bacterial community composition and snail life history. Our data fill in data gaps regarding gut microbes in Viviparidae and highlight future research paths examining the prevalence of Firmicutes and unidentified diversity in both snail species.

KEY WORDS: bacteria, microbial communities, *Campeloma*, *Cipangopaludina*, next-generation sequencing

INTRODUCTION

Animal gut bacteria are critical for the health of their hosts; they affect nutrition, behavior, immune responses, and development (Uzbay 2019). This has been demonstrated in snails as well, where gut microbes are ubiquitous contributors to many physiological processes. Snail gut bacteria play crucial roles in digesting organic material and producing nutrients (Hu et al. 2018). They break down structural carbohydrates such as cellulose, chitin, and lignin, and they provide nitrogen and organic precursors for the production of nucleic acids and the metabolism of energy (Nicolai et al. 2015; Pinheiro et al. 2015; Aronson et al. 2017). Up to 80% of plant-derived carbohydrates are broken down by bacterial enzymes that augment the snail's own digestive enzymes (Charrier et al. 2006). Gut bacteria also have been shown to differ between sexual and asexual populations of the same snail species, suggesting a microbial aspect to reproduction (Takacs-Vesbach et al. 2016).

Multiple factors are known to influence the composition and function of the animal gut microbiome. Bacterial communities can vary significantly between individuals and between species. Diet, geography, season, and disease have been shown to cause variation in—and potential disruption of—a host's gut flora (Colman et al. 2012; King et al. 2012;

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Tang et al. 2019). Gut microbes also are subject to spatial and temporal differences throughout the host lifecycle (Llewellyn et al. 2016; Triplett et al. 2020). Three planorbid snail species exhibited significant differences in gut microbial diversity and abundance at both the individual and species levels (Van Horn et al. 2012). Different compartments of the digestive system of *Achatina fulica* possessed different microbial communities; these same communities differ further between active and estivating individuals (Pawar et al. 2012). The addition of sugarcane to the diet of *A. fulica* changed the taxonomic composition of the gut microbiome (Cardoso et al. 2012).

Despite recent efforts, relatively little is known about freshwater snail gut microbiomes (Hu et al. 2018; Lyra et al. 2018; Li et al. 2019; Huot et al. 2020). Viviparidae comprises operculate live-bearing snails whose females brood their young in a pouch formed from the palatal oviduct. The Pointed Campeloma, *Campeloma decisum*, is native to the USA, ranging from the Great Lakes and Mississippi River drainages east to the Atlantic slope (Clench 1962). *Cipangopaludina japonica*, the Japanese Mysterysnail, likely was introduced to North America from Asia in the late 19th century by food vendors and spread through intentional and accidental means (Wood 1892; Rothlisberger et al. 2010). In the USA, the species is widespread, reaching its highest density in the Great Lakes and northeastern states (Perez et al. 2016). *Campeloma decisum* and *Ci. japonica* occupy similar habitats and ecological niches: individuals are frequently found on soft sediments in rivers and lakes, and both species are presumed to filter feed from the water column as well as ingest organic material present in the substrate (Allison 1942; Chamberlain 1958; Bocxlaer and Strong 2016).

To explore the diversity and function of these important systems, we studied the gut microbial communities of two viviparid snails. We were interested in testing three central predictions regarding the gut microbes in *Ca. decisum* and *Ci. japonica*. First, we predicted that estimates of alpha and beta diversity would not differ significantly between the snail species given their similar environmental and ecological niches. Second, we predicted that those similarities in diversity would correspond to nonsignificant differences in the estimated bacterial group abundances between the two species. Finally, we predicted that similar *Ca. decisum* and *Ci. japonica* bacterial communities would possess similar estimated microbiome functions. Our ultimate goal was to determine how knowledge of their gut microbial communities affects our understanding regarding the biology and life history of these two species.

METHODS

We acquired 14 *Cameploma decisum* and 13 *Cipangopaludina japonica* from a single collection event at a single site on the Flat River in Lowell, Michigan (42.934° N, 85.339° W) in August 2017. Snails were frozen live at –80°C after collection. After removing the bodies from the shells, we rinsed the animals in deionized water and dissected the intestines and posterior portion of the stomachs. Following the manufacturer's directions, we used the DNeasy PowerSoil (Qiagen) kit to extract microbial genomic DNA from the tissues. We sent the DNA samples to MrDNALab (Shallowater, TX), where the bacterial 16S V4 region was amplified by PCR using the 515F-806R primer pair (Caporaso et al. 2011) and sequenced on an Ion Torrent PGM (Thermo Fisher). Ion Torrent methods produce unidirectional reads of approximately 250 bp by using forward and reverse sequencing primers that are subsequently assembled. MrDNALab performed all quality control on the sequencing output using their proprietary pipeline. Briefly, sequences were depleted of primers; short sequences ($<$ 150 bp) and sequences with ambiguous base calls were removed. Sequences were quality-filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated sequences were denoised; unique sequences identified by sequencing or PCR point errors were removed, as were chimeras; and ends were trimmed.

We used QIIME2 (Bolyen et al. 2019) to analyze the assembled reads. We de-replicated our sequences (vsearch option) and removed any amplified sequence variants (ASVs) present in fewer than two snail samples and/or with abundances below 10 reads summed across all samples (McDonald et al. 2012; Bokulich et al. 2013; Rognes et al. 2016; Takacs-Vesbach et al. 2016). We used the align-to-tree-mafft-fasttree pipeline to perform multiple sequence alignment of our ASVs, mask ambiguously aligned regions, and build maximum likelihood trees (Price et al. 2010; Katoh and Standley 2013). We compared three measures of ASV alpha diversity between *Ca. decisum* and *Ci. japonica*: Shannon diversity as a quantitative estimate of community richness (Shannon 1948), Faith's PD as a qualitative estimate of community richness incorporating phylogenetic relatedness (Faith 1992), and Pielou's index as a measure of community evenness (Pielou 1966). For beta diversity comparison between the snail species, we utilized a permutational multivariate analysis of variance (PERMANOVA) based on generalized UniFrac distances (Anderson 2001; Lozupone and Knight 2005; Chen et al. 2012). Generalized distances combine presence-absence and abundance data with phylogenetic distances between ASVs in the computations, while adjusting the weighting on the branches (Chang et al. 2011). Beta diversity was also assessed at a sampling depth of 5,600 ASVs. Significance of all between-species measures was determined using Kruskal-Wallis tests at $P < 0.05$. We additionally visualized relationships between individual snails using UPGMA based on generalized UniFrac distances in QIIME2.

We hierarchically classified our ASVs using QIIME2 and a pretrained naïve Bayesian classifier based on the 99% OTU Greengenes 13_8 database (DeSantis et al. 2006; Pedregosa et al. 2011; Bokulich et al. 2018). Each ASV was classified to the lowest phylum, family, and genus assigned by the classifier, and abundances were compared between snail species. For unclassified groups that differed significantly between snail species, we selected 50 random ASVs from each group and used the NCBI blastn tool (Altschul et al. 1990) with default

Table 1. Alpha diversity statistics for individual viviparid snails. Cd, *Campeloma decisum*; Cj, *Cipangopaludina japonica*; ASVs, amplified sequence variants.

Snail	Raw reads	ASVs	Shannon diversity	Faith's PD	Pielou's evenness
Cd1	63,884	29,325	4.18	5.13	0.45
Cd2	82,596	61,399	5.58	3.77	0.57
Cd3	75,356	53,078	5.83	7.75	0.58
Cd4	48,528	30,732	6.46	7.27	0.64
Cd5	64,572	38,370	6.96	7.03	0.68
Cd6	63,046	28,933	6.62	9.89	0.67
Cd7	67,309	45,817	6.26	11.75	0.62
Cd8	70,799	41,512	5.41	6.21	0.57
Cd9	38,322	19,989	6.29	13.50	0.66
Cd10	62,095	38,213	6.60	8.24	0.65
Cd11	59,945	38,340	6.35	8.30	0.63
Cd12	16,784	5,642	6.68	7.75	0.70
Cd13	72,397	39,562	3.32	5.65	0.42
Cd14	52,784	25,131	5.71	4.19	0.61
Cj1	58,969	36,240	4.66	8.12	0.50
Cj2	59,771	39,162	4.99	6.82	0.54
Cj3	78,576	52,858	5.63	8.79	0.58
Cj4	75,647	44,210	5.09	5.25	0.53
Cj5	81,376	61,783	5.49	4.42	0.56
Cj6	27,526	10,008	6.43	13.15	0.72
Cj7	80,157	45,606	7.15	5.40	0.71
Cj8	59,666	38,299	5.74	11.20	0.59
Cj9	65,165	38,069	6.07	4.90	0.64
Cj10	35,717	20,102	5.40	10.48	0.58
Cj11	66,937	39,112	6.67	5.21	0.67
Cj12	38,265	19,800	4.67	11.55	0.53
Cj13	64,135	35,494	6.78	15.28	0.67

settings to assess similarity compared to sequences accessioned in GenBank. Finally, we predicted the functional composition of each snail species' gut microbial metagenome using PICRUSt2 (Douglas et al. 2020). PICRUSt2 reconstructs a simulated metagenome from the samples provided, then predicts the function of the metagenome through comparison to the prokaryotic portion of the MetaCyc database (Caspi et al. 2018). ASVs aligning with less than 80% similarity to the reference sequences were excluded, as were those exceeding a nearest sequenced taxon index of 2.0, based on the default settings in PICRUSt2. Significant taxonomic and functional differences between species were identified in ALDEx2 (Fernandes et al. 2014). ALDEx2 uses centered log ratios to convert absolute feature counts to relative abundances normalized for sequencing effort modeled from a Dirichlet process (Holmes et al. 2012; Rosa et al. 2012). Significant differences were assessed by ALDEx2 using estimated *P*-values from Welch's *t*-tests controlled for Benajmini-Hochberg false-discovery rates (FDR) less than 0.1 (Welch 1947; Benjamini and Hochberg 1995).

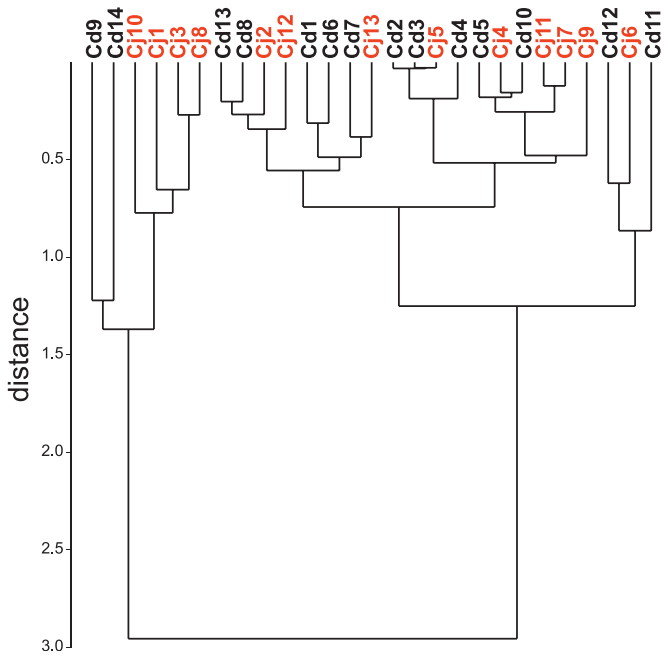

Figure 1. UPGMA dendrogram of pairwise generalized UniFrac distances between individual snails. Black labels, *Campeloma decisum*; red labels, *Cipangopaludina japonica*.

RESULTS

A total of 1,630,324 raw reads were generated for all 27 snails examined, yielding 976,786 total ASVs after dereplicating and filtering. Mean raw reads and ASVs for *Ca. decisum* were 59,887 and 35,432, respectively, and 60,916 and 36,980 for *Ci. japonica*. There was no significant difference in mean ASV number recovered between species (*t*-test, $t = 0.29$, $P = 0.77$). Neither Shannon diversity, nor Pielou's evenness, nor Faith's PD were significantly different between species (Shannon, $H = 0.53$, $P = 0.47$; Pielou, $H = 0.19$, $P = 0.66$; Faith, $H = 0.24$, $P = 0.63$; Table 1). Beta diversity assessed through PERMANOVA of generalized UniFrac distances also did not differ between species (999 permutations, $F = 1.78$, $Q = 0.14$). A UPGMA based on generalized UniFrac distances showed that samples from each snail species did not cluster together (Fig. 1).

Firmicutes and Proteobacteria were the most abundant bacterial phyla in both *Ca. decisum* and *Ci. japonica*, while Bacteroidetes had a mean abundance 100× higher in *Ci. japonica*. Nine families were identified as occurring at $\geq 1\%$ relative abundance in at least one snail species, with Bacillaceae being most abundant in both. Enterobacteriaceae was recovered from only *Ca. decisum*. *Bacillus* was the most abundant genus identified in both snails, with five genera occurring at $\geq 1\%$ relative abundance in at least one snail species. Gut-microbe mean relative abundances are summarized in Figure 2. At the family level, five classifications were significantly more abundant in *Ca. decisum*: families Pseudomonadaceae, Enterobacteriaceae, Mycobacteriaceae, and Staphylococcaceae and order Rhizobiales unclassified to

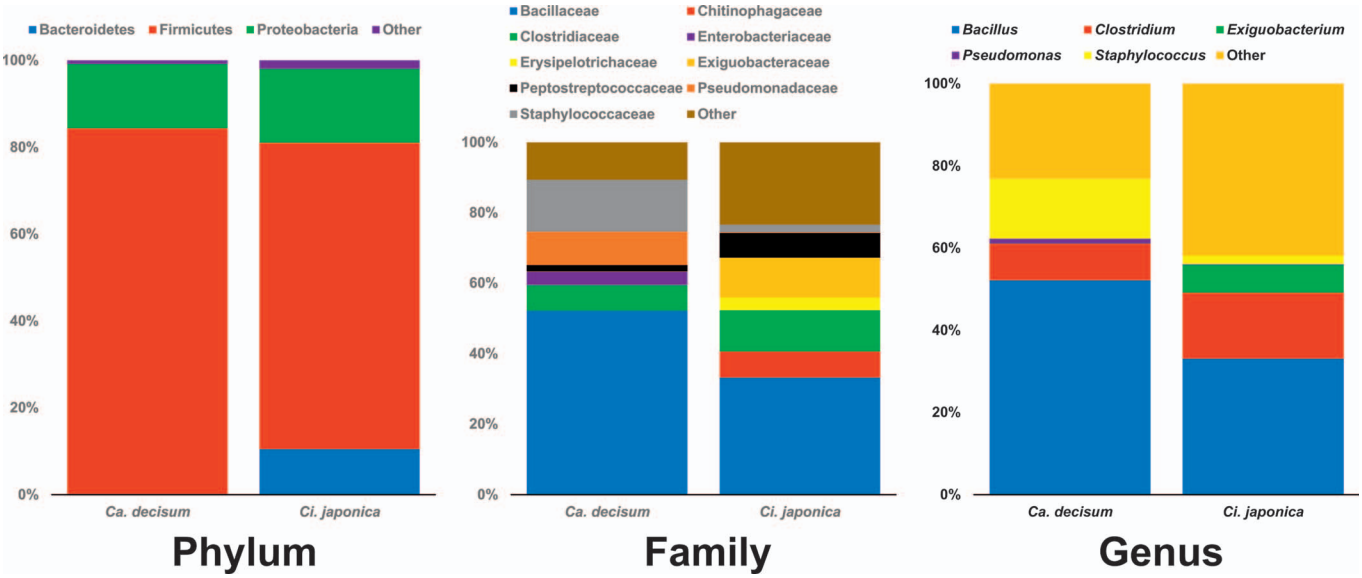

Figure 2. Mean gut-microbe relative abundances between *Campeloma decisum* and *Cipangopaludina japonica*. Values are mean relative percent abundance by snail species. Classification is the lowest hierarchical level that ASVs were assigned to and had relative abundances $\geq 1\%$ in at least one species; the remaining groups are placed in "Other."

family. In *Ci. japonica*, two classifications were significantly more abundant: bacteria unclassified beyond kingdom Bacteria and the phylum Proteobacteria. Analysis of genus-level classifications indicated similar differences compared to those seen at the family level, with the addition of bacteria unclassified beyond family Chitinophagaceae being more abundant in *Ci. japonica*. Comparisons between the snail species with *P*-values, FDR values, and effect sizes are shown in Table 2.

From the unclassified past kingdom and past family Proteobacteria in *Ci. japonica*, 50 ASVs each were compared against GenBank using blastn. All ASVs from the unclassified kingdom group had the same best match, a *Mycoplasma* sp. isolated from *Biomphalaria glabrata* (GenBank accession

Table 2. Significant gut-microbe differences between *Campeloma decisum* and *Cipangopaludina japonica*. Analysis indicates at which taxonomic level the two species were compared. Classification is the lowest hierarchical level that ASVs were assigned in the comparison. Species column reflects in which species the bacterial group was detected at the significantly higher relative abundance. Statistics are the estimated *P*-values derived from Welch's *t*-tests, Benjamini-Hochberg false discovery rates (FDR), and effect sizes. Significance was measured at FDR $\alpha < 0.1$.

Analysis	Classification	Species	*P*	FDR	Effect size
Phylum	Kingdom Bacteria	*Ci. japonica*	0.0130	0.076	0.836
Family	Family Pseudomonadaceae	*Ca. decisum*	0.0009	0.024	0.891
	Family Enterobacteriaceae	*Ca. decisum*	0.0097	0.071	0.755
	Family Mycobacteriaceae	*Ca. decisum*	0.0076	0.049	0.972
	Family Staphylococcaceae	*Ca. decisum*	0.0018	0.032	0.894
	Order Rhizobiales	*Ca. decisum*	0.0080	0.064	0.812
	Kingdom Bacteria	*Ci. japonica*	0.0118	0.073	0.835
	Phylum Proteobacteria	*Ci. japonica*	0.0001	0.005	1.371
Genus	Family Pseudomonadaceae	*Ca. decisum*	0.0005	0.017	1.089
	Family Enterobacteriaceae	*Ca. decisum*	0.0109	0.087	0.773
	Genus *Mycobacterium*	*Ca. decisum*	0.0088	0.067	0.931
	Genus *Staphylococcus*	*Ca. decisum*	0.0034	0.060	0.801
	Order Rhizobiales	*Ca. decisum*	0.0093	0.086	0.800
	Genus *Pseudomonas*	*Ca. decisum*	0.0091	0.096	0.691
	Kingdom Bacteria	*Ci. japonica*	0.0123	0.091	0.812
	Family Chitinophagaceae	*Ci. japonica*	0.0051	0.056	0.953
	Phylum Proteobacteria	*Ci. japonica*	0.0001	0.008	1.371

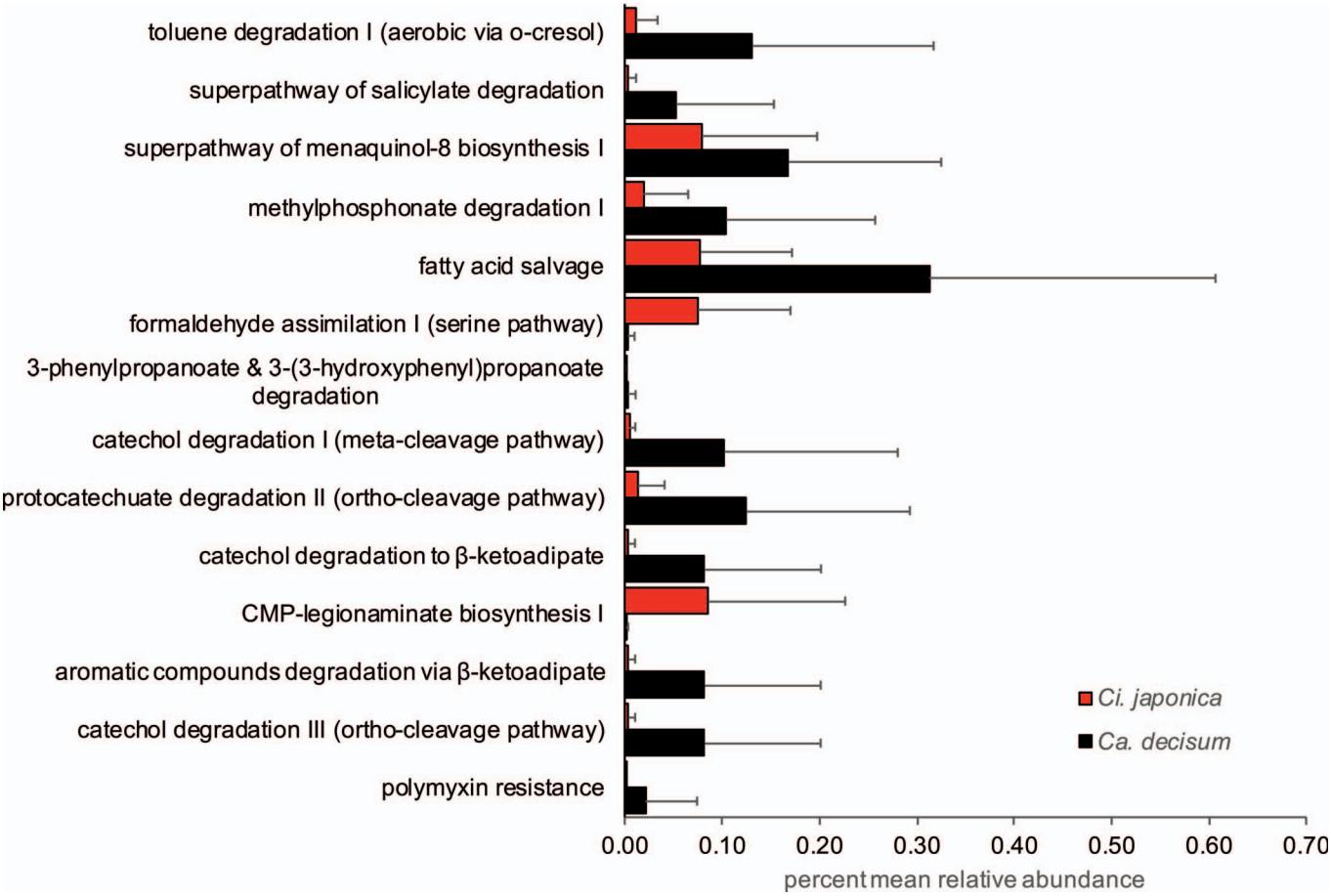

Figure 3. Functional pathway abundances for *Campeloma decisum* and *Cipangopaludina japonica*. Only those pathways that differed significantly (FDR < 0.1) between snail species are shown. Error bars represent one standard deviation.

number CP013128). All ASVs from unclassified Proteobacteria matched best to an unclassified Gammaproteobacteria isolated from the gut of *Achatina fulica* (JN211207).

PICRUSt2 analysis of predicted metagenomes identified peptidoglycan synthesis, pyruvate fermentation, and aerobic respiration by cytochrome *c* as the three most abundant microbial pathways represented in the viviparid gut; they were the only pathways to occur at greater than 1% relative abundance in each snail species. Significant differences in predicted function between *Ca. decisum* and *Ci. japonica* were detected in 14 pathways (Fig. 3). CMP-legionaminate biosynthesis and formaldehyde assimilation pathways were more abundant in *Ci. japonica*. In *Ca. decisum*, degradation pathways (aromatic compounds, catechol, methylphosphonate, proponoate, protocatechuate, salicylate, toluene), fatty acid salvage, menaquinol-8 biosynthesis, and polymyxin resistance pathways were more abundant.

DISCUSSION

Given the importance of gut microbes in an animal's life history and the paucity of knowledge regarding the gut flora of freshwater snails, we aimed to better understand the gut microbial communities in *Ca. decisum* and *Ci. japonica*. Our results represent the first efforts to estimate the diversity and predicted function of gut bacteria in Viviparidae and the second examination of gut flora from a freshwater snail species native to North America (Van Horn et al. 2012). Our data supported our first prediction that *Ca. decisum* and *Ci. japonica* would possess significantly different alpha and beta diversities. Microbiome estimates from both species were not significantly different in terms of ASV richness, evenness, or phylogenetic diversity. Additionally, beta diversity measured by generalized UniFrac distances did not significantly differ between samples from the two species. In general, gut microbiomes arise from two main sources: vertical parental transmission and horizontal environmental acquisition (Rothschild et al. 2018). Research has consistently shown that the environment plays the largest role in shaping gut microbial communities (Preheim et al. 2011; Schmidt et al. 2019). Since our snail samples were taken from the same locality during the same collection event—and given their similar known ecologies—we were unsurprised to find no significant differences between diversity measures in *Ca. decisum* and *Ci. japonica*.

In both *Ca. decisum* (84.24%) and *Ci. japonica* (70.58%),

bacteria in Firmicutes showed the highest relative abundance, with Proteobacteria being the next most abundant (14.75% and 17.02%, respectively). Proteobacteria have been observed to be the most abundant microbial phylum in the gut of terrestrial, marine, and freshwater snails (Pawar et al. 2012; Lyra et al. 2018; Ito et al. 2019). Of the snail species whose gut flora have been studied, the majority are either herbivores or periphyton grazers/scrapers (e.g., *Achatina fulica*, *Biomphalaria glabrata*, *Radix auricularia*, and *Batillus cornutus*). Rare exceptions exist, including the deep sea, bone-eating *Rubyspira osteovora* and the generalist *Pomacea canaliculata* (Johnson et al. 2010; Oosterom et al. 2016). In our viviparid samples, Firmicutes were most abundant. Members of this group have been found in low abundance among snail gut microbes and are slightly more abundant in freshwater species (Takacs-Vesbach et al. 2016; Lyra et al. 2018; Huot et al. 2020). Firmicutes do, however, compromise a major component (up to 64%) of the gut flora associated with soil-dwelling invertebrates, including earthworms, isopods, springtails, and millipedes (König 2006). Given that *Ca. decisum* and *Ci. japonica* filter feed and process detritus from sediment, their diets may include food sources more similar to those found in soil habitats than to herbivores or periphyton ingesters. Within Firmicutes, Bacillaceae and *Bacillus* were the most abundant in the two viviparid species. This suggests that the diets of *Ca. decisum* and *Ci. japonica* contain many organic plant polymers, including cellulose and hemicellulose. *Bacillus* are capable of digesting available carbohydrates and recalcitrant biological materials, such as chitin and lignocellulose (König et al. 2006). *Clostridium* (Clostridiaceae) were also abundant in both snails' digestive tracts, further reflecting a diet heavy in plant polysaccharides (Boutard et al. 2014).

Significant differences in relative microbial abundance were observed for several bacterial groups in *Ca. decisum* and *Ci. japonica* and, thus, did not support our second prediction. In *Ca. decisum*, Pseudomonadaceae and *Pseudomonas* were two of the more abundant groups. Pseudomonads are better known from the gut flora of terrestrial snails than freshwater snails (Nicolai et al. 2015; Takacs-Vesbach et al. 2016; Hu et al. 2018) and may participate in the anaerobic hydrolysis of plant carbohydrates (Buettner et al. 2019). Enterobacteriaceae, Staphylococcaecae, and *Staphylococcus* were also more abundant in *Ca. decisum*. Enterobacteriaceae are commonly found in animal gut microbiota, where they ferment sugars to lactic acid and other products; in addition, most can reduce nitrate to nitrite (Octavia and Lan 2014). Staphylococci are known from the digestive systems of freshwater fish and mussels but are rarely represented in snails (Jami et al. 2015; Weingarten et al. 2019). The role of these bacteria in the animal gut is poorly known, though they may provide mechanisms for hydrocarbon breakdown (Kayath et al. 2019).

In studies of animal gut microbes, many bacteria remain unclassified by the methods employed or are able to be classified only at higher taxonomic levels (Thomas and Segata 2019). This was the case for *Ci. japonica*, where bacteria that could not be classified past kingdom and those that could not be classified past Proteobacteria were significantly more abundant relative to *Ca. decisum*. Using blastn, we were able to match subsets of each group to microbes isolated from other snail taxa. All 50 ASVs from the unclassified bacterial kingdom group matched to a *Mycoplasma* sp. isolated from the freshwater planorbid *Biomphalaria glabrata*. We found it interesting that *Mycoplasma* was detected in *Ci. japonica* but not in *Ca. decisum. Mycoplasma* species are well-characterized intracellular animal parasites (Razin et al. 1998). Invasive species may harbor bacteria from their native range, but they also develop novel associations with microbes found where they are introduced (Bankers et al. 2020). While present in *Ci. japonica*, these unclassified, *Mycoplasma*-like bacteria were present in low numbers (0.34% mean abundance). We hypothesize that *Ci. japonica* may be more susceptible to *Mycoplasma* infection in its introduced range than the indigenous *Ca. decisum*. All 50 ASVs from unclassified Proteobacteria were most similar to an unclassified Gammaproteobacteria isolated from the gut of the land snail *Achatina fulica* (Pawar et al. 2012). Gammaproteobacteria are common animal gut microbes, and the unclassified ASVs suggest the presence of novel bacterial taxa from the family in *Ci. japonica*.

Gut bacteria comprise both those microbes that live in the animal host symbiotically and those that are ingested as food or ingested nonselectively through feeding. We found groups that were likely ingested by the snails, given that they are considered environmental taxa and not present in animal digestive systems. *Cipangopaludina japonica* had a high, but not significantly different, relative abundance of *Exiguobacterium* (Exiguobacteraceae). These bacteria are ubiquitous in soil and freshwater and have been shown to break down a variety of plant carbohydrates (Kasana and Pandey 2018). Chitinophagaceae were significantly more abundant in *Ci. japonica*. As their name implies, these bacteria can hydrolyze chitin from the environment and are found primarily in soils and aquatic sediments (Lim et al. 2009; Madhaiyan et al. 2015). They are often poor hydrolyzers of plant carbohydrates such as cellulose and starch, but they can ferment sugars into organic acids (Sangkhobol and Skerman 1981). Their abundance in *Ci. japonica* may be a result of untested dietary or microhabitat differences between snail species. Bacteria from the order Rhizobiales were significantly more abundant in *Ca. decisum*. Rhizobiales are frequently associated with plants; some taxa are nitrogen-fixing bacteria associated with the rhizosphere, while others are intracellular pathogens (Delmotte et al. 2009). Mycobacteriaceae and *Mycobacterium* were also significantly more abundant in *Ca. decisum*. These are ubiquitous soil bacteria and not gut flora (Pontiroli et al. 2013). The relative abundance differences of these in *Ca. decisum* versus *Ci. japonica* suggest diet or microhabitat differences between the two snails, as the snails are ingesting and processing different materials.

Our data also failed to support our third prediction, since significant differences were observed in the predicted microbial community functions of *Ca. decisum* and *Ci.*

japonica, although in pathways of low abundance (1.65% of total abundance summed across functions). In *Ca. decisum*, degradation pathways for aromatic compounds, catechol, protocatechuate, and salicylate are all associated with *Pseudomonas* species that were significantly more abundant (e.g., Chan et al. 1979; Harayama and Rekik 1990; Díaz 2004). These degradation pathways are interconnected mechanisms for bacteria to break down environmental pollutants such as phthalates, hydroxybenzoates, and toluene (Parales and Harwood 1993; Przybylińska and Wyszkowski 2016). The fatty acid salvage pathway also appears to be an additional means for *Pseudomonas* to produce long-chain fatty acids (Yuan et al. 2012). Phenylpropanoate degradation, a pathway for the breakdown of aromatics in Proteobacteria (Burlingame and Chapman 1983), was also more abundant in *Ca. decisum*. We hypothesize that the significant abundance of *Pseudomonas* and degradation pathways were the result of snail hosts adapting to environmental contamination. While the Flat River is considered a relatively "healthy" river, agricultural runoff, septic systems, and other human activities are thought to be pollution sources (Michigan Department of Environmental Quality 2006). These activities generate significant amounts of aromatic contaminants that can end up in freshwater sediments (Malaj et al. 2014). By harboring more pseudomonads and Proteobacteria that can degrade and metabolize the contaminants, *Ca. decisum* may increase their survivorship in polluted fresh waters. *Cipangopaludina japonica* may possess other pathways to deal with contamination or may not be able to host native pseudomonads as readily as *Ca. decisum.*

Other functional pathways that lacked clear correlations to the environment were more abundant in *Ca. decisum*. Menaquinol-8 biosynthesis, methylphosphonate degradation, and polymyxin resistance all showed higher abundance in *Ca. decisum*. Menaquinols function in the bacterial electron chain; they also participate in the production of vitamin K2 for their animal hosts (Meganathan 2001). Phosphonate degradation provides bacteria with an alternate source of phosphorous in addition to the breaking of phosphoester bonds of phosphates (Huang et al. 2005). Polymixins are polypeptides produced by Gram-positive Bacilliaceae that disrupt the outer membrane of Gram-negative bacteria. These results may suggest that *Ca. decisum* has a different requirement for vitamin K2 than *Ci. japonica* and possibly needs alternate sources of phosphorous. The presence of polymyxin resistance may be tied to the increased abundance in *Pseudomonas*, which are known to acquire resistance and may require it to persist in the same microbiome with polymyxin-producing Bacilliaceae (Tam et al. 2005).

Two bacterial pathways were more abundant in the predicted metagenomes from *Ci. japonica*. The formaldehyde assimilation pathway is used by methanotrophic bacteria to oxidize methane into formaldehyde, which can be used to form intermediates needed for other metabolic pathways (Quayle and Ferenci 1978). The increased presence of methane oxidation implies that *Ci. japonica* is directly or indirectly ingesting more material from anaerobic sediment than *Ca. decisum*. While both species burrow into the sediment, this finding may suggest that *Ci. japonica* spends more time burrowed or burrowed deeper than *Ca. decisum* (Szal and Gruca-Rokosz 2020). CMP-legionaminate biosynthesis was also more abundant in *Ci. japonica*. This pathway is involved in sialic acid metabolism, one means by which pathogenic bacteria can avoid the host immune system (Schoenhofen et al. 2009). Abundance of the pathway may correlate with the potential presence of *Mycoplasma* in *Ci. japonica* and not *Ca. decisum*. The binding of *Mycoplasma* to host cells is modulated by sialic acid residues (Nishikawa et al. 2019) and may explain why the pathway is significantly more abundant in *Ci. japonica*.

Our results highlight limitations and paths for future research on freshwater snail gut microbes. Next-generation, high-throughput sequencing methods have become the standard for exploring microbial diversity using 16S sequences (Poretsky et al. 2014). Our data were generated using Ion Torrent chemistry, an older method that generates fewer sequence reads per sample with higher error rates. Illumina technology is seen as superior, generating higher numbers of more accurate reads, though each method biases its results differently (Salipante et al. 2014). While both methods generate statistically consistent taxonomic and functional microbial profiles when read numbers are similar (Onywera and Meiring 2020), the increase in read number from Illumina methods may provide more adequate sampling of the gut flora. Also, our use of the intestine and partial posterior stomach for analysis may have been suboptimal, since separate digestive compartments possess their own set of microbes (Pawar et al. 2012). We were able to minimize some variation in our data by using snails collected from the same location at the same time but did not tightly control for how much stomach was used. We also did not assess the microbial diversity of the water and sediment at the collection site, so our determinations of enteric versus environmental taxa may be skewed or incorrect. Although they were from the same site, it is unknown whether the two snail species occupied identical microhabitats. Subtle differences in microhabitats may be reflected in the significant differences in low-abundance groups and functions (Fiore et al. 2020). Finally, our results indicated that Firmicutes were the major component of viviparid gut flora. This finding is in sharp contrast to all other mollusks whose gut microbes have been assessed. Determining why Firmicutes were the dominant phylum and not others, namely Proteobacteria, would allow a better integrative approach to understanding viviparid diet, metabolism, habitat usage, and life history. More complete pathway analysis and biochemical testing would also test hypotheses of taxon and pathway abundances that differ between species.

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