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HOST MICROBE INTERACTIONS

Characterization of the Cutaneous Bacterial Communities of Two Giant Salamander Subspecies

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Abstract Pathogens currently threaten the existence of many amphibian species. In efforts to combat global declines, researchers have characterized the amphibian cutaneous microbiome as a resource for disease management. Characterization of microbial communities has become useful in studying the links between organismal health and the host microbiome. Hellbender salamanders (*Cryptobranchus alleganiensis*) provide an ideal system to explore the cutaneous microbiome as this species requires extensive conservation management across its range. In addition, the Ozark hellbender subspecies (*Cryptobranchus alleganiensis bishopi*) exhibits chronic wounds hypothesized to be caused by bacterial infections, whereas the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) does not. We assessed the cutaneous bacterial microbiome of both subspecies at two locations in the state of Missouri, USA. Through 16S rRNA gene-based amplicon sequencing, we detected more than 1000 distinct operational taxonomic units (OTUs) in the cutaneous and environmental bacterial microbiome. Phylogenetic and abundance-based dissimilarity matrices identified differences in the bacterial communities between the two subspecies, but only the abundance-based dissimilarity matrix identified differences between wounds and healthy skin on Ozark hellbenders. The higher abundance of OTUs on Ozark wounds suggests that commensal bacteria present on the skin and environment may be opportunistically colonizing the wounds. This brief exploration of the hellbender cutaneous bacterial microbiome provides foundational support for future studies seeking to understand the hellbender cutaneous bacterial microbiome and the role of the bacterial microbiota on chronic wounds of Ozark hellbenders.

Introduction

Amphibian declines due to disease (such as chytridiomycosis and Ranavirus infections) have gained a great deal of attention within the past 20 years, and recent analyses have attempted to characterize the microbiome on the skin of amphibians as a way to link microbiome composition and disease susceptibility [1–3]. Next-generation DNA sequencing technologies have greatly increased the efficiency of microbial ecology studies. These technologies have opened up the possibility to characterize the microbiome of a diverse array of habitat types. Recent studies exploring bacterial communities, their functions, and their influence on their habitat have revolutionized our understanding of the microbial world in general. For example, microbiome characterization as a way to study disease in wildlife now includes studies characterizing the fecal [4], skin [5], respiratory system [6], and even whole colony microbiota [7]. Because disease has become a major factor of worldwide amphibian decline [8], expanding the use of this technology to amphibian taxa is crucial for future conservation efforts and decisions.

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Hellbenders (*Cryptobranchus alleganiensis*) are fully aquatic salamanders that have a range spanning the eastern USA. These salamanders are restricted to lotic habitats that are fast flowing, cool, and have abundant rock cover [9]. In the past 20 years, this species has been facing population declines throughout the range associated with habitat loss, water quality disruptions, harvesting, and disease [10–13]. The eastern hellbender subspecies (*Cryptobranchus alleganiensis alleganiensis*) is broadly distributed throughout the Appalachian Mountain region and also the Midwest including Kentucky, Ohio, Indiana, Illinois, and Missouri. Eastern hellbenders are now classified as threatened or endangered in many of these states. The Ozark hellbender subspecies (*Cryptobranchus alleganiensis bishopi*) is endemic to southern Missouri and northern Arkansas and has also suffered population declines and local extirpations. Because of their limited distribution and population sizes, Ozark hellbenders are now federally listed under the Endangered Species Act [14, 15]. The state of Missouri is the only state within the range of hellbenders where both subspecies are present, though not sympatric.

Ozark hellbenders currently experience disease caused by unknown pathogen(s), as manifested by chronic cutaneous wounds throughout the body [16–18]. While ranaviruses and amphibian chytrid fungus have not been associated with these wounds, a bacterial pathogen has been suspected [17, 19]. Nickerson et al. [17] cultured and identified bacteria and fungi from six Ozark hellbender wounds showing signs of repressed tissue regeneration. The authors were able to identify representatives of a total of four bacterial and two fungal phyla from which some bacterial isolates were categorized as opportunistic pathogens. However, certain colonies were not identifiable and some inoculates exhibited no growth, as many bacteria cannot grow in culture mediums [20, 21]. Thus, they likely identified only a small subset of the total microbial diversity within the wounds of Ozark hellbenders.

Unlike Ozark hellbenders, chronic skin lesions have not been observed on eastern hellbenders in Missouri (*C. a. alleganiensis*) (J. Briggler, unpublished data). This key difference provides an ideal opportunity to explore the cutaneous microbiome between the two subspecies. While fungi and viruses could also be found on the skin of hellbenders, we chose to concentrate on the exploration of the cutaneous bacterial communities based on suspected bacterial involvement in hellbenders [17] and amphibians in general [1, 22–25]. The threatened status of hellbenders, and the presence of chronic wounds in only one subspecies, creates an opportunity to comparatively explore the cutaneous bacterial communities using a culture-independent approach (eg, next-generation sequencing). Next-generation sequencing technologies and statistical tools are more sensitive in the detection and quantification of rare species in microbial communities [26]. Assessing the bacterial communities between the intact skin and wounds of Ozark hellbenders could provide relevant information regarding the colonization of wounds by opportunistic bacteria. In addition, understanding the structure of the microbial communities between the two hellbender subspecies could inform future conservation decisions.

In the present study, we characterize the bacterial microbiomes of the two hellbender subspecies and between healthy skin and wounds of Ozark hellbenders using next-generation sequencing. When comparing Ozark and eastern hellbenders, we predicted that subspecies' identity should be the primary driver of the bacterial microbiome composition on the skin of these salamanders. In addition, microbiome communities will differ based on Ozark hellbender swab location (ie, wound or healthy skin).

Materials and Methods

Sample Collection

Hellbenders were sampled from November 5 to 6, 2013. Samples for the eastern subspecies were collected from the Niangua River in central Missouri and the Ozark subspecies from the North Fork of the White River in southern Missouri. All individuals were captured by hand and rinsed with 1 L of sterile water. Rinsing the animal prior to swabbing was intended to ensure that the sample primarily included skin-associated microorganisms as opposed to those associated with the water column [27]. Following rinsing, the hellbenders were swabbed with sterile cotton-tipped swabs (Medline Industries Inc., Mundelein, Illinois) for 30 s in a rotating motion while applying minimum pressure on the skin. From each hellbender, two healthy skin samples were collected: one from the dorsum and one from the plantar surface of one foot free of wounds. Sample swabs from all wounds present on the hellbender's body were also collected. Immediately after collection, the sample swabs were stored in 1.5-mL microcentrifuge tubes, placed on dry ice, and moved to a −80 °C freezer within 48 h. All hellbenders were returned to their location of capture within the river following sampling. River water samples were obtained from each locality by collecting 4 L of water from each river 1 to 10 m upstream from where hellbender sampling occurred. Water samples were stored on dry ice for up to 12 h until filtration occurred in an aseptic environment using a Whatman glass microfiber filter (GE Healthcare, Chicago, IL). The filters were stored in 15-mL centrifuge tubes and placed in a −80 °C freezer until DNA isolation occurred.

DNA Extractions, Amplification, and Sequencing

The two river samples were extracted using a PowerWater DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) following their protocol for organisms that are difficult to lyse. Extraction of the swab samples was performed using a

PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) with a modified protocol (found in Supplementary Material, p. 1). The 16S rRNA gene V2 region was amplified for bacteria by using the primer set 27F/338R as in Fierer et al. [28]. Library preparation was performed in two sequential PCRs per sample, one in which a combination of the amplifying primers and partial Illumina adaptors were attached (performed in duplicate for each sample) and a second one in which dual-index barcodes were attached to each end with the remaining portion of the sequencing adaptors (Table S2). The initial PCRs consisted of 5 μL of template DNA, 5 μL of 5xMyTaq Reaction Buffer (Bioline, Tauton, MA, USA), 0.5 μL of MyTaq DNA Polymerase (Bioline, Tauton, MA, USA), 1 μL of 10 mM forward and reverse primers, and 13.5 μL of PCR water (MoBio Laboratories, Carlsbad, CA, USA) for a total of 25 μL per reaction. PCR conditions for these reactions were 95 °C for 2 min, 30 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, followed by 72 °C for 10 min. Following the initial PCR, the duplicate PCR products were pooled and cleaned using an UltraClean PCR Clean-Up kit (MoBio Laboratories, Carlsbad, CA, USA) following manufacturer's protocol. The second PCR consisted of 5 μL of clean PCR product from the initial PCR, 5 μL of 5xMyTaq Reaction Buffer, 0.5 μL of MyTaq DNA Polymerase, 1 μL of 10 mM forward and reverse barcoded primers (specific per sample), and 13.5 μL of PCR Water for a total of 25 μL. PCR conditions consisted of 95 °C for 2 min, 10 cycles of 94 °C for 45 s, 65 °C for 60 s, and 72 °C for 90 s, followed by 72 °C for 10 min. PCR products were quantified using a Qubit Fluorometer (Invitrogen, NY) and pooled in equimolar amounts to be cleaned using the UltraClean PCR Clean-Up kit. The cleaned sample pool was sent to the Purdue Genomics Core Facility and sequenced on one lane of the Illumina HiSeq 2500 (Illumina Inc., San Diego, CA, USA) as 150 bp paired-end reads in rapid mode.

Sequence and Statistical Analysis

Resulting reads were processed using Trimmomatic [29] to remove Illumina adapter sequences and low quality bases (below threshold quality of phred-20) from both ends of the reads. The reads were further processed using a custom Python program (Supplementary Material, p. 13) that removed any reads fewer than 150 bp in length, to standardize read length, and renamed the reads with a name compatible with our chosen pipeline. The forward read file was processed using the Quantitative Insights Into Microbial Ecology version 1.8.0 (QIIME) pipeline [30]. Reads were grouped into operational taxonomic units (OTUs) at the standard 97 % identity using UCLUST [31]. To avoid including any OTUs generated by sequencer errors, such as base miscalls or chimeras, OTUs that were represented by a frequency fewer than 0.005 % of the total reads or were absent from more than one

sample were removed [32]. The most abundant read per OTU was chosen as the representative sequence for that OTU in later analyses. Representative sequences were aligned to the pre-aligned Greengenes 13_5 release database [33] using PyNAST [34] at a minimum percent identity match of 75 %. Taxonomy was assigned to each OTU representative sequence using the UCLUST consensus taxonomy assigner and the Greengenes 13_5 OTU database at 80 % confidence [35]. The aligned representative OTU sequences were then used to generate a phylogenetic tree through FastTree to be used in later phylogenetic based distance matrix calculations [36]. Trimmomatic filtered reads and metadata were deposited in the European Bioinformatics Institute (primary accession no. PRJEB9832). OTUs were assigned and quality filtered from all environmental and animal samples. Following OTU assignment, the generated OTU table was rarified to 40,030 sequences per sample based on the sample with the lowest read count. To evaluate the diversity differences between the two river samples, OTU relative abundances were compared between the two river samples by computing a Pearson correlation coefficient.

Alpha diversity metrics (Chao 1, phylogenetic diversity index, Shannon-Wiener index, and observed species) for each eastern dorsum, eastern foot, Ozark dorsum, and Ozark wound sample were calculated within the QIIME pipeline. All four metrics were included to account for richness and evenness differences in alpha diversity between samples. Chao 1 is a richness estimator based on the number of rare classes found in samples [37]. The phylogenetic diversity index is a richness measure that uses the minimum total length of the phylogenetic branches represented by the bacterial species in a sample [38]. Finally, the Shannon-Wiener index is a quantitative measure that accounts for the frequency that each bacterial species is observed within a sample (community diversity) [39]. Calculated alpha diversity metrics were uploaded to R for subsequent analysis.

Feet Versus Dorsum Community Comparisons

To test for alpha diversity differences between sample types in eastern (dorsum versus feet) and Ozark hellbenders (dorsum versus wounds), a linear mixed model was applied through the package *lme4* [40]. Sample type was included as a fixed variable and individual identity as a random variable. Beta diversity (using the unweighted UniFrac, weighted UniFrac, and Bray-Curtis dissimilarity metrics) was calculated independently for eastern and Ozark hellbender samples to produce between-sample dissimilarity matrices within QIIME. The UniFrac metrics use the phylogenetic distances between observed OTUs in each community when calculating distance between communities based on presence/absence (unweighted) or abundance (weighted) [41]. Alternatively, the Bray-Curtis

metric incorporates OTU abundance only in the calculation of dissimilarity between samples [42]. Unweighted UniFrac and Bray-Curtis dissimilarity matrices were used to perform principal coordinate analysis (PCoA) in QIIME. To test for community differences between sample types for Ozark and eastern hellbenders, the dissimilarity matrices were uploaded to R. The dissimilarity matrices were used to perform a two-way Adonis test using both individual identity and sample types as grouping variables under the package *vegan* [43]. Additionally, a two-way PERMANOVA was calculated for wound versus dorsum Ozark hellbender comparisons with individual identity as a second variable in Past 3.10 [44].

To derive the OTUs that differentiate between Ozark hellbender dorsum and wounds, the core OTUs present in 95 % of samples in each sample type were first identified. This strict cutoff was chosen as it has been applied in other studies with low sample sizes, ensuring that only common OTUs represented across sample types are included [45]. Venn diagrams were created to display the number of core OTUs shared between sample types using the package *VennDiagram* in R. The linear discriminant analysis effect size (LEfSe) algorithm described in Segata et al. [46] was used to test for significant differences in core OTU relative abundance between dorsum and wounds. The LEfSe algorithm identifies the OTUs whose abundance statistically differs between the subspecies through a nonparametric factorial Kruskal-Wallis sum rank test ($\alpha < 0.05$). Subsequently, generated effect sizes are calculated through a linear discriminant analysis (LDA) for those divergent OTUs. The effect sizes represent the magnitude of the association of each relevant core OTU to the category assigned. Because taxonomy data was not inputted into LEfSe, the resulting LDA scores reflect core OTU differences only. Core OTU taxonomy data was retrieved from Greengenes assignments performed in QIIME with an additional search of sequences in the Ribosomal Database Project (RDP) to confirm taxonomy or resolve unassigned sequences [47].

Subspecies Community Comparisons

Subspecies comparisons of alpha diversity metrics were performed using a Student's *t* test since each sample derived from a unique individual. Beta diversity (using the unweighted UniFrac, weighted UniFrac, and Bray-Curtis dissimilarity metrics) was calculated for eastern hellbender dorsum and Ozark hellbender dorsum samples to produce between-sample dissimilarity matrices within QIIME. Unweighted UniFrac and Bray-Curtis dissimilarity matrices were used to perform the PCoA's. To test for community differences between subspecies, the dissimilarity matrices were uploaded to R and used to calculate one-way ANOSIM and Adonis tests

with subspecies identity as a grouping variable. Core OTUs present in 95 % of all Ozark and eastern dorsum samples were identified independently for each subspecies. The lists were used to produce Venn diagrams of shared core OTUs between both subspecies, inputted into LEfSe to test for differences in core OTU relative abundance between subspecies, and used to identify taxonomy as described above.

Results

We sampled five unique eastern and six unique Ozark hellbenders providing us with ten eastern healthy skin swabs, 11 Ozark healthy skin swabs, and nine Ozark wound swabs (Table S1). We removed one Ozark hellbender foot sample (9F) from the analysis due to a low number of sequences after the filtering process ($n = 304$). From each river, we collected one water sample. After assessing for quality, the filtered sequence set analyzed contained a total of 15,590,201 filtered reads of ~150 bp length that were used in the remaining analyses. Our OTU list comparisons for the two river water samples showed high similarity (Pearson correlation value of $r = 0.94$ with a $p < 0.001$).

Feet Versus Dorsum Community Comparisons

Alpha metrics (Table S3) between eastern hellbender dorsum and feet samples were comparable (Table 1). The two-way Adonis test performed on the dissimilarity matrices produced for the feet and dorsum of eastern hellbenders showed no significant community differences for sample type (unweighted UniFrac: $R = 0.08$, $p = 0.321$; weighted UniFrac: $R = 0.08$, $p = 0.182$; Bray-Curtis: $R = 0.08$, $p = 0.159$) but significant differences between individuals (unweighted UniFrac: $R = 0.65$, $p = 0.023$; weighted UniFrac: $R = 0.74$, $p = 0.008$; Bray-Curtis: $R = 0.75$, $p = 0.002$). PCoAs generated from beta diversity metrics display no evident pattern between eastern foot and dorsum samples (Fig. S2). Finding no differences between sample types in healthy eastern hellbenders allowed us to continue comparisons between the healthy dorsum of eastern and Ozark hellbenders and between the Ozark dorsum and wound samples.

Alpha metric comparisons between Ozark hellbender dorsum and wound swabs revealed that wound sample richness outnumbered dorsum richness (Table 1 and Fig. 1). However, Shannon-Wiener and phylogenetic diversity comparisons between Ozark dorsum and wound swabs were comparable (Table 1). Community comparisons between Ozark hellbender wound and dorsum swabs using the two-way Adonis tests resulted in differences between sample type and individuals for both unweighted UniFrac (sample type $R = 0.10$, $p = 0.019$; individual $R = 0.47$, $p = 0.004$) and Bray-Curtis distance matrices (sample type $R = 0.17$, $p = 0.001$; individual $R = 0.53$,

Table 1 Alpha diversity comparisons between hellbender (*Cryptobranchus alleganiensis*) subspecies

Alpha diversity comparisons

	Shannon	Chao1	Observed OTUs	Phylogenetic diversity
Eastern dorsum skin versus Ozark dorsum skin	$t_{8.63} = 0.43$ $p = 0.675$	$t_{8.03} = -0.43$ $p = 0.676$	$t_{8.18} = -0.54$ $p = 0.603$	$t_{7.67} = -0.22$ $p = 0.828$
Ozark dorsum skin versus wounds	LRT = 3.36 $p = 0.067$	LRT = 6.90 $p = 0.008$	LRT = 4.34 $p = 0.037$	LRT = 2.82 $p = 0.093$
Eastern dorsum skin versus feet	LRT = 0.44 $p = 0.506$	LRT = 2.69 $p = 0.101$	LRT = 0.92 $p = 0.337$	LRT = 0.67 $p = 0.412$

For inter-subspecies comparisons (Ozark versus eastern hellbenders), results for Student's *T* tests on four different diversity metrics are presented. For intra-subspecies comparisons (Ozark dorsum skin versus wounds and eastern dorsum skin versus feet), linear mixed model results are presented on four different alpha diversity metrics. Individual identity was included in the linear mixed model as a random variable and sample origin as a fixed variable

$p = 0.007$). The two-way Adonis test on the weighted UniFrac distance matrix between Ozark wound and dorsum swabs returned no differences between sample type but differences between individuals (sample type $R = 0.06$, $p = 0.337$; individual $R = 0.50$, $p = 0.034$). The two-way PERMANOVA comparisons revealed significant differences between Ozark sample type and individuals for the Bray-Curtis distance matrix (type: pseudo-$F = 0.84$, $p = 0.023$; individual: pseudo-$F = 0.53$, $p = 0.050$; interaction: pseudo-$F = -0.32$, $p = 0.962$) and non-significant differences for the unweighted UniFrac (type: pseudo-$F = 0.81$, $p = 0.061$; individual: pseudo-$F = 0.76$, $p = 0.062$; interaction: pseudo-$F = 0.03$, $p = 0.278$) or weighted UniFrac distance matrices (type: pseudo-$F = 0.18$, $p = 0.663$; individual: pseudo-$F = 0.30$, $p = 0.448$; interaction: pseudo-$F = -0.36$, $p = 0.961$). These results indicate that community dissimilarity between Ozark dorsum and wound swabs is due to differences in the abundance of shared OTUs observed within each sample type. Our PCoA plots comparing Ozark hellbender dorsum skin and wound swab communities support our statistical results by displaying no distinct grouping by sample type for coordinates calculated from the unweighted UniFrac distance matrix

(Fig. 2b), but distinct grouping from the Bray-Curtis distance matrix (Fig. 3b).

Subspecies Community Comparisons

Alpha metrics (Table S3) between hellbender subspecies dorsum samples were comparable (Table 1). We noted community differences between the sampled eastern and Ozark hellbender dorsum communities for all metrics testing with both Adonis (unweighted UniFrac: $R = 0.25$, $p = 0.024$; weighted UniFrac: $R = 0.26$, $p = 0.019$; Bray-Curtis $= 0.26$, $p = 0.011$) and ANOSIM tests (unweighted UniFrac: $R = 0.34$, $p = 0.020$; weighted UniFrac: $R = 0.39$, $p = 0.006$; Bray-Curtis $= 0.48$, $p = 0.008$). Our PCoA plots comparing both hellbender subspecies dorsum swabs show distinct grouping by subspecies identity for coordinates derived from unweighted UniFrac (Fig. 2a) and Bray-Curtis distance matrices (Fig. 3a). These results suggest that the cutaneous microbiome of eastern and Ozark hellbenders differs in both composition and abundance of OTUs.

Core Bacterial Microbiome Results

Our core microbiome cutoff identified 70 core OTUs from eastern hellbender dorsum skin swabs, 112 core OTUs from Ozark hellbender dorsum skin swabs, and 321 core OTUs from Ozark hellbender wound swabs. There was an overlap in the core OTUs identified between Ozark and eastern hellbenders dorsum and Ozark hellbender dorsum and wound samples (Fig. 4). Phylum-level richness of the core bacterial microbiome of eastern and Ozark hellbenders was similar, while the wound core bacterial microbiome contained higher richness with a larger percentage of unclassified bacteria (Table S4).

The LEfSe algorithm identified 117 distinct core OTUs between Ozark and eastern hellbenders (Table S5). Thirty-seven OTUs were associated with eastern hellbenders,
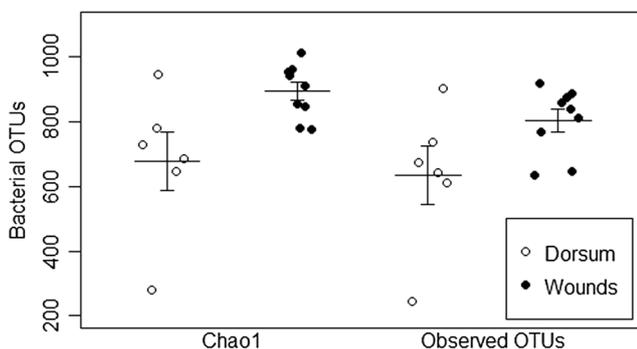

Fig. 1 Dot plot of estimated (*Chao1*) and observed species alpha diversity metrics for bacterial communities collected from Ozark hellbender dorsum and wound swabs. Group means and standard error bars are presented

Fig. 2 Principal coordinate analyses (PCoAs) of unweighted UniFrac distances. **a** Comparison between dorsum swabs of eastern (*Cryptobranchus alleganiensis alleganiensis*) and Ozark (*C. a. bishopi*) hellbenders. Ozark dorsum and wound swabs are compared in **b**
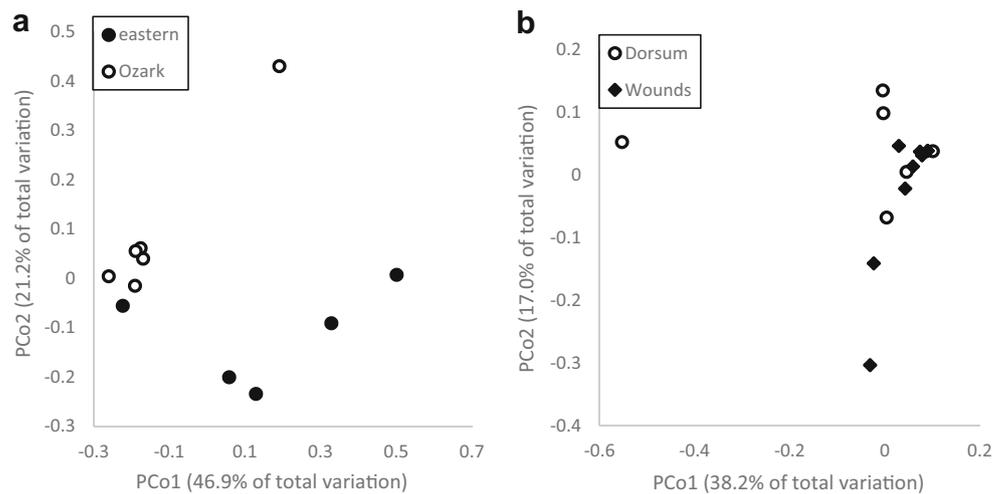
whereas 80 were associated with Ozark hellbenders. OTUs identified as *Cetobacterium somerae*, *Leptospira* sp., *Bacteroides* sp., *Alistipes* sp., and an unclassified species in the family *Enterobacteriaceae* were assigned with high LDA effect sizes to eastern hellbenders (>4.0). OTUs identified as *Bacillariophyta* sp., an unclassified species in the family *Sphingomonadaceae*, *Dechloromonas* sp., an unclassified species in the class *Betaproteobacteria*, *Massilia* sp., and *Novosphingobium* sp. received the highest associations to the Ozark hellbenders (LDA>3.2). The LEfSe algorithm identified 29 core OTUs to be discriminant biomarkers between wounds and healthy dorsum swabs collected from Ozark hellbenders, with 26 wound-associated and 3 dorsum-associated bacteria (Table 2). Four OTUs with unknown taxonomy (denovo206854, denovo37779, denovo40873, and denovo84139) were associated with wounds and exhibited high relative abundance across wound swabs. For OTUs whose taxonomy is known *Acidovorax* sp., *Flavobacterium* sp., *Aquabacterium* sp., *Chryseobacterium* sp., and *Acinetobacter* sp. also displayed high effect size and high relative abundance within wounds compared to intact skin.

Discussion

Microbiome studies across diverse amphibian taxa provide evidence for an association between hosts and their microbial symbionts [5, 49]. Our study is unique among other amphibian cutaneous microbiota projects because it builds on a previous culture-based approach characterizing the wounds of Ozark hellbenders in the North Fork of the White River, MO [17]. Compared to the work performed by Nickerson et al. [17], the number of bacterial OTUs described in this culture-independent study is significantly greater. We also included samples from eastern hellbenders and intact skin from the Ozark hellbenders from which we obtained wound samples. This allowed us to compare the bacteria of wounds to healthy skin sites and of affected to healthy populations. From our samples, we were able to identify unclassified, culture-specific, and unculturable bacteria [48], which could not be identified using the methods of Nickerson et al. [17].

The two hellbender subspecies sampled in our study differed in their skin-associated bacterial communities (Figs. 2a and 3a). Although the differences observed could be due to

Fig. 3 Principal coordinate analyses (PCoAs) of Bray-Curtis distances. **a** Comparison between dorsum swabs of eastern (*Cryptobranchus alleganiensis alleganiensis*) and Ozark (*C. a. bishopi*) hellbenders. Ozark dorsum and wound swabs are compared in **b**
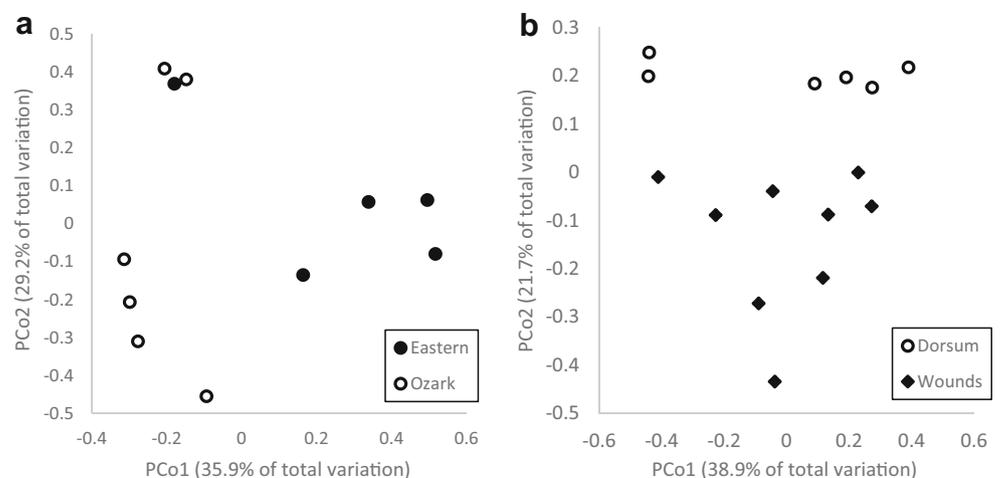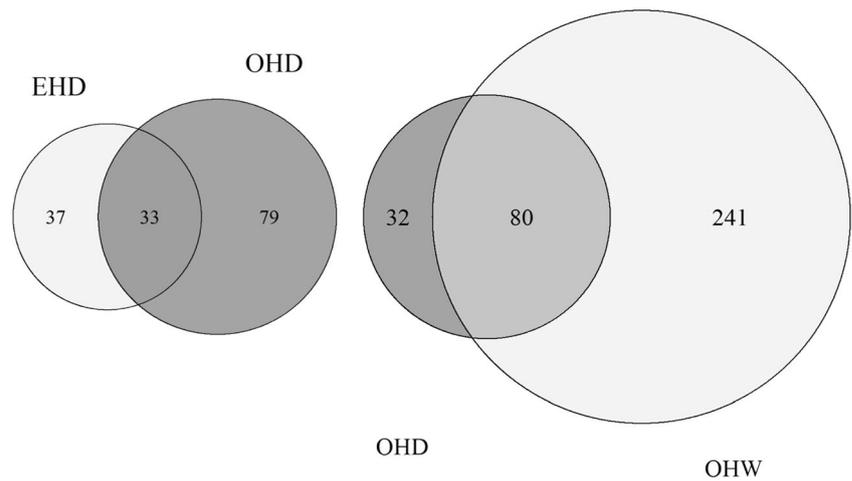
Fig. 4 Venn diagrams comparing the dorsum skin core microbiome of eastern (*EHD*) and Ozark (*OHD*) hellbenders (*left*) and the dorsum and wounds (*OHW*) of Ozark hellbenders (*right*)

other factors (e.g., difference in age, sex, exposure to different sediment bacteria), the results of this study are consistent with previous bacterial microbiome evaluations on the skin of amphibians [5, 49–51]. The cutaneous bacterial communities of amphibians have been strongly associated with host species identity; thus, indicating that skin bacterial communities are specific to each amphibian species [5, 49]. In hellbenders, phylogenetic assessments across the species range offer strong support for paraphyly for the two currently known subspecies [52, 53]. Through this perspective, differences in the bacterial microbiome between the subspecies could be driven by the same speciation forces already acting between them [54].

However, these observations are based on individuals from two proximate rivers within the large range of the species and could be due to environmental differences between the two streams. While the communities that we identified from the river water were similar, they do not reflect all environmental sources of bacteria (i.e., substrate). Thus, characterizing the skin and environmental bacterial microbiome throughout the entire range of hellbenders will allow to test if symbiotic differences follow patterns similar to those of genetic differentiation between host populations [55].

While Ozark and eastern hellbenders shared a subset of their core OTUs, the Ozarks displayed a higher OTU richness

Table 2 Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) dorsum and wound LEfSe assigned OTUs, Taxonomy, LDA scores, significance values, and relative abundance for respective swab IDs. Cell color is assigned based on relative abundance value from 0 (white) to 0.1 (black)

		OTU ID	Taxonomy	LDA	p	06D	07D	09D	10D	11D	12D	06W	07W	09W	10W	11W	12WB	12WL	12WLF	12WRF
LEfSe assignment	Wounds	denovo40873	Unknown bacteria	3.46	0.001															
		denovo84139	Unknown bacteria	3.32	0.001															
		denovo37779	Unknown bacteria	3.13	0.001															
		denovo47610	Unknown bacteria	2.85	0.001															
		denovo36572	Genus *Acinetobacter*	2.85	0.018															
		denovo177597	Genus *Acidovorax*	2.84	0.001															
		denovo226124	Genus *Flavobacterium*	2.71	0.001															
		denovo124611	Order Burkholderiales	2.57	0.001															
		denovo90520	Genus *Deefgea*	2.52	0.001															
		denovo107521	Unknown bacteria	2.46	0.001															
		denovo40522	Unknown bacteria	2.46	0.001															
		denovo279129	Genus *Aquabacterium*	2.45	0.001															
		denovo42208	Unknown bacteria	2.42	0.001															
		denovo160390	Unknown bacteria	2.37	0.001															
		denovo26217	Unknown bacteria	2.30	0.001															
		denovo206854	Unknown bacteria	2.26	0.001															
		denovo119424	Genus *Deinococcus*	2.22	0.001															
		denovo140176	Unknown bacteria	2.20	0.001															
		denovo284734	Unknown bacteria	2.12	0.001															
		denovo33763	Order Stramenopiles, class Bacillariophyta	2.11	0.001															
		denovo174986	Order Stramenopiles, class Bacillariophyta	2.08	0.001															
		denovo234736	Genus *Porphyrobacter*	2.07	0.001															
		denovo244042	Phylum Saccharibacteria	2.07	0.001															
		denovo179894	Genus *Steroidobacter*	2.06	0.001															
		denovo70045	Genus *Pseudomonas*	2.04	0.001															
		denovo253431	Unknown bacteria	2.03	0.001															
	Dorsum	denovo146716	Order Stramenopiles, class Bacillariophyta	2.59	<0.001															
		denovo79105	Genus *Propionibacterium*	2.40	<0.001															
		denovo208984	Order Streptophyta	2.21	<0.001															

than the easterns (Fig. 4). These core OTUs are specific to the populations sampled, and it is unknown whether they reflect the core bacterial microbiota of each subspecies across the range. Therefore, investigating the bacterial communities of hellbenders across the range of both subspecies could also reduce the list of core OTUs. Identifying the subset that is common between all localities where these salamanders are found could provide insight to current conservation approaches. Captive rearing and translocations have become common conservation tools for hellbenders across the range [56, 57], and these methods have been associated with disruption of the natural microbiome of amphibians [58]. Given that bacterial symbionts protect the amphibian host against pathogens [25], maintaining diversity of the core microbiome may increase the survivorship of reintroduced individuals. Thus, it is important to minimize the disruption of the microbiome when moving individuals from source to supplemented populations. The composition of our hellbender skin bacterial microbiome is comparable to that of other North American amphibians and fish (Fig. S1) [49, 51, 59], suggesting a close association between bacterial symbionts and these taxa across evolutionary time. Similarities between hellbenders compared to other aquatic vertebrate taxa and the existence of an extensive captive rearing effort in this species, suggest that future investigations in wild and captive hellbenders could provide knowledge applicable to conservation of other amphibians.

Wound samples contained higher OTU richness than dorsum samples within Ozark hellbenders (Table 1 and Fig. 1), while community structure differed between the two sample types based on abundance only (Figs. 2b and 3b). The differences in OTU richness and community structure observed between these sample types can be attributed to the pathophysiology of the chronic wounds. Adult hellbenders tend to acquire injuries during the mating season, when territorial bouts occur between conspecifics or as a result of predator attacks [16, 60]. While eastern hellbenders are able to heal completely as evidenced by a lack of chronic wounds, Ozark hellbenders are unable to clear their injuries retaining an open port of colonization [18]. We cannot determine whether the OTUs that we identified in wounds are the cause of wound retardation in this subspecies, but we can note that these open areas provide a novel habitat that commensal and environmental bacteria can exploit. As a result, the exposed tissue could provide a habitat through which a richer community is maintained compared to that of the skin.

Apart from differences in OTU richness between Ozark hellbender wounds and dorsum skin, we were also able to identify OTUs that differed in their abundance between both sample types (Table 2). These wound-assigned OTUs were identified to the genus *Acidovirax* within the family *Comamonadaceae*, the genus *Flavobacterium* within the family *Flavobacteriaceae*, and the genus *Acinetobacter* within the family *Moraxellaceae*. These families have been previously described as common members of the amphibian skin microbiome [5, 24], indicating that the OTUs identified on the wounds could derive from the healthy skin. *Acidovorax* sp. has been recovered from the skin of other amphibians [61–63], including identification on Italian stream frogs (*Rana italica*) infected with amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) [1]. The genus *Flavobacterium* has been recovered from several amphibian species and has been suspected to possess protective roles in the microbiome of the amphibian host [1, 61, 63, 64]; however, the same genus has been associated with dermatosepticemia in amphibians [65–67]. *Acinetobacter* sp. has been recovered from skin lesions and inner organs of the hellbender's sister species, *Andrias davidianus*, in individuals that perished from *Ranavirus* infections [68]. While our methodology makes it difficult to declare these wound-associated species as pathogens or protective symbionts, it is likely that some of these OTUs contribute to the chronic wound status. Therefore, future bacterial evaluations on hellbenders and other amphibians should consider these OTUs (Table 2) as they could correlate with host health status.

We found differences in the bacterial community between the two subspecies of hellbenders that coincide with the divergence of these two groups. Our findings serve as a starting point towards future microbiome assessments in this species, which could inform the conservation of hellbenders and other amphibians. The negative effects of disease on amphibians plus the concomitant linkage between the cutaneous bacterial microbiome and host health drive the need to utilize these taxa as a model for microbiome assessment. The current focus of amphibian bacterial microbiota studies resides mostly within the realm of chytridiomycosis resistance research [22, 69]. Susceptibility to chytridiomycosis has been found to be mediated in part by members of the skin bacterial communities, and current conservation approaches to mediate disease include probiotic applications [70]. However, we have shown that high-throughput techniques can be employed to assess the bacterial microbiota between groups of amphibians which are not related to devastating diseases.

Compliance with Ethical Standards

Conflict of Interest   The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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