EcoHealth 15, 109–120, 2018
DOI: 10.1007/s10393-017-1289-y

EcoHealth

Original Contribution

Common Cutaneous Bacteria Isolated from Snakes Inhibit Growth of *Ophidiomyces ophiodiicola*

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Abstract: There is increasing concern regarding potential impacts of snake fungal disease (SFD), caused by *Ophidiomyces ophiodiicola* (*Oo*), on free-ranging snake populations in the eastern USA. The snake cutaneous microbiome likely serves as the first line of defense against *Oo* and other pathogens; however, little is known about microbial associations in snakes. The objective of this study was to better define the composition and immune function of the snake cutaneous microbiome. Eight timber rattlesnakes (*Crotalus horridus*) and four black racers (*Coluber constrictor*) were captured in Arkansas and Tennessee, with some snakes exhibiting signs of SFD. *Oo* was detected through real-time qPCR in five snakes. Additional histopathological techniques confirmed a diagnosis of SFD in one racer, the species' first confirmed case of SFD in Tennessee. Fifty-eight bacterial and five fungal strains were isolated from skin swabs and identified with Sanger sequencing. Non-metric multidimensional scaling and PERMANOVA analyses indicated that the culturable microbiome does not differ between snake species. Fifteen bacterial strains isolated from rattlesnakes and a single strain isolated from a racer inhibited growth of *Oo* in vitro. Results shed light on the culturable cutaneous microbiome of snakes and probiotic members that may play a role in fighting an emergent disease.

Keywords: Dermatophytic fungi, Wildlife disease, Emerging fungal pathogens, Cutaneous microbiome

Introduction

Anthropogenic effects, such as habitat fragmentation and climate change, have been linked with an increase in newly emerging fungal pathogens contributing to significant population declines in a broad diversity of wildlife (Pechmann and Wilbur 1994; Lips 1998; Daszak et al. 1999; Houlahan

Electronic supplementary material: The online version of this article (https://doi.org/10.1007/s10393-017-1289-y) contains supplementary material, which is available to authorized users.

Published online: November 13, 2017

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et al. 2000; Daszak et al. 2003; Smith et al. 2006; Woodhams et al. 2008; Rödder et al. 2009; Fisher et al. 2012). Notably, the causative agent of white-nose syndrome in bats, *Pseudogymnoascus destructans,* has been linked to mass mortalities of bat species across the eastern USA (Blehert et al. 2009; Gargas et al. 2009). Amphibian population declines have been exacerbated by the globally distributed pathogen *Batrachochytrium dendrobatidis* and the newly described *Batrachochytrium salamandrivorans* in Europe (Bosch et al. 2001; Garner et al. 2006; Belden and Harris 2007; Rödder et al. 2009; Martel et al. 2014). In reptiles, anthropogenic impacts, such as habitat loss, introduction of invasive species, environmental pollution, and disease, are causing population declines in numerous species (Hinton and Scott 1990; Case and Bolger 1991; Alford and Richards 1999). Recently, there are increasing concerns regarding potential population-level impacts of an emerging mycotic infection known as snake fungal disease (SFD; Clark et al. 2011; Allender et al. 2015c; Paré and Sigler 2016).

Clinical signs of SFD include cutaneous lesions, such as crusty scales, ulcers, and occasional granulomas of the eyes and lungs (Allender et al. 2011; Dolinski et al. 2014; Guthrie et al. 2015). The causative agent of SFD has recently been confirmed using Koch's postulates as *Ophidiomyces ophiodiicola* (*Oo*; Allender et al. 2015a; Lorch et al. 2015). This fungus was originally described in a captive black rat snake in 2009 from Georgia and has since been documented in wild populations in at least 17 states and provinces of North America, including Arkansas, Florida, Georgia, Illinois, Louisiana, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, North Carolina, Ohio, Ontario, Tennessee, Virginia, West Virginia, and Wisconsin (Allender et al. 2015c). Documented free-ranging hosts include the Eastern massasauga (*Sistrurus catenatus*), plains garter snake (*Thamnophis radix*), timber rattlesnake *(Crotalus horridus),* black racer (*Coluber constrictor*), black rat snake (*Elaphe obsoleta obsoleta),* broad-banded water snake (*Nerodia fasciata confluens),* rainbow snake (*Farancia erytrogramma),* mud snake (*Farancia abacura),* and brown water snake (*Nerodia taxispilota)* (Rajeev et al. 2009; Clark et al. 2011; Dolinski et al. 2014; McBride et al. 2015; Guthrie et al. 2015; Tetzlaff et al. 2015; Glorioso et al. 2016; Last et al. 2016). Diagnostic techniques vary from study to study, and confident diagnoses can be difficult to obtain (Allender et al. 2015a; Lorch et al. 2015). Paré and Sigler (2016) highlight the importance of genotype-based identification of fungal isolates, alongside culture-based and pathological techniques, in order to properly diagnose SFD.

The cutaneous microbiome is known to serve as the first line of pathogen defense in a diverse range of vertebrate species (Harris et al. 2006; Lauer et al. 2007; Bletz et al. 2013; Park et al. 2014; Smeekens et al. 2014; Woodhams et al. 2014); however, very little is known about the immune function of symbiotic microbes living on snakes. Resident bacterial species in the reptilian cutaneous microbiome may affect the pathogenicity of *Oo* by outcompeting the pathogen for space, producing antifungal metabolites, and/or stabilizing the microbial community to increase defensive efficacy, all of which have been documented in amphibians (Harris et al. 2006; Lauer et al. 2007; Bletz et al. 2013; Park et al. 2014; Woodhams et al. 2014). The use of antifungal bacteria or their metabolites is not a novel concept. In fact, bioaugmentation has been used effectively in marine and freshwater aquaculture for over twenty years (Gil-Turnes and Fenical 1992; Verschuere et al. 2000; Irianto and Austin 2002). Currently, there is growing experimental support for probiotic use in wildlife conservation of threatened species (Harris et al. 2009; Cornelison et al. 2014; Woodhams et al. 2016), and its efficacy has been demonstrated in vitro (Lauer et al. 2007). Identification of beneficial probiotic bacteria on snake skin will aid in our understanding of microbial community dynamics and their tentative effect on pathogenicity.

We hypothesize that the reptilian cutaneous microbiome may affect the manifestation of disease due to infection with *Oo*. To allow for future testing of this hypothesis, we conducted a foundational study characterizing the snake cutaneous microbiome and the potential ability of commensal bacterial species to inhibit *Oo* growth. Specifically, the objectives of this project were to (1) isolate culturable skin microbiota from two snake species in the southeastern USA, (2) identify bacteria and fungi with DNA sequencing, (3) compare the culturable microbiome between snake species with and without *Oo* infection, and (4) determine whether resident bacteria possess antifungal activity when challenged in vitro against *Oo*.

Methods

Sample Collection

Between October 2015 and October 2016, eight free-ranging timber rattlesnakes (n = 8; *Crotalus horridus*) were captured from four counties in Tennessee and one county in Arkansas. Four of the rattlesnakes exhibited signs of SFD,

but the other individuals appeared healthy. Also during this time, four free-ranging black racers (n = 4; *Coluber constrictor*) were captured from four Tennessee counties, with two of these racers presenting with signs of SFD. Three snakes with cutaneous lesions were brought to the laboratory for sampling, and the remaining snakes were sampled in the field and released. All snakes were handled with clean, nitrile gloves and transient microbes rinsed with 100 mL of sterile deionized water that had been autoclaved for 2 h (Lauer et al. 2007; Gefrides et al. 2010; Walker et al. 2015). Sterile rayon-tipped swabs (Puritan; VWR cat #10808-146) were rolled over a small surface area of rinsed skin ($\approx$ 15 cm in length), either healthy skin on the mid-lateral surface of snakes without lesions, or lesions if present, for 15 strokes. Two skin swabs were collected. The first swab was stored in a sterile, dry 2-mL tube for later extraction of total genomic DNA, and the second swab was stored in a sterile 20% glycerol tube for later bacterial and fungal isolation. Samples were promptly frozen and stored at − 20°C.

Cutaneous Microbiome Sampling and Bioinformatics

For isolation of microbes from the cutaneous microbiome, skin swabs were thawed and streaked across agar plates using a standard microbiological streak plating technique. Fungi were isolated in pure culture by streaking the skin swabs across potato dextrose agar (PDA) and corn meal agar (CMA) plates containing 100 mg/mL each of neomycin sulfate, erythromycin, penicillin G, and ampicillin. Plates were stored at ambient room temperature under 24-h fluorescent light and checked daily for growth. When visible fungal hyphae grew, they were isolated onto secondary PDA plates and grown for 2–5 days for DNA extraction. To isolate bacteria, swabs were streaked across tryptic soy agar (TSA) plates and incubated at 32°C. All morphologically distinct colonies were isolated onto secondary TSA plates. Primary plates were monitored for 7 days or until no new colonies arose.

Fungal DNA was extracted using the MoBio PowerFecal Kit (MoBio Laboratories, Inc., Carlsbad, California), and the ITS rDNA marker was PCR-amplified using primers ITS4 and ITS5 (White et al. 1990) as in Walker et al. (2010, 2012). PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, California) and DNA sequenced (see below). Pure bacterial colonies were grown on fresh TSA agar for 24 h, and direct colony was PCR-amplified with 16S rRNA primers 515F and 806R (Caporaso et al. 2012). Each PCR had a total volume of 25 μL and contained 2.5 μL of 10X reaction buffer, 0.5 μL of 10 μM dNTPs, 0.5 μL of 10 μM of each primer, 0.15 μL of VersaTaq Direct PCR Polymerase (Affymetrix), 20.85 μL of sterile water, and a single bacterial colony aseptically added to the PCR. PCR conditions were as follows: initial denaturation for 10 min at 95°C, followed by 30 cycles of 45 s at 94°C, 60 s at 50°C, and 90 s at 72°C, and a final extension period of 10 min at 72°C. Electrophoresis on a 1% agarose gel was used to separate PCR products, and $\sim$ 250–300 bp fragments were excised and purified using the EZNA Gel Extraction Kit (Omega Bio-tek, Norcross, GA).

Sequencing was completed at MC Lab on an ABI 3730XL sequencer under standard cycling conditions. Resulting nucleotide sequences were edited using ChromasPro (Technelysium, South Brisbane, Queensland, Australia) and compared to sequence databases for Sanger sequencing purposes. Fungal species identification was accomplished using the UNITE ver. 7.0 database (Kõljalg et al. 2013) with a cutoff value of 98.5% identity to the closest species hypothesis. Bacterial 16S sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the command pick_open_reference_otus.py, implemented in Quantitative Insights into Microbial Ecology (QIIME; Caporaso et al. 2010). Bacterial taxonomic identification was accomplished in QIIME using Greengenes reference set ver. gg_13_8_otus and the command assign_taxonomy.py.

Challenge Assays

A single isolate of *Oo* (#12-34933; Allender et al. 2015c) was grown in pure culture on PDA for 28 days under ambient conditions and 24-h fluorescent light for challenge assays. Standardized aliquots of homogenized mycelium, including conidia, were prepared in separate Falcon tubes for each challenge plate as follows: An approximately 2 cm^2 piece of mycelium was scraped off the agar, sliced into small pieces using a sterile scalpel, and placed into a 50-mL Falcon tube containing 10 sterile 4-mm silica beads and 1.0 mL sterile water. The mycelium was homogenized by completing two replicates of grinding with a sterile glass rod for 2 min, followed by vortexing at 3000 rpm for 2 min. Wide-bore pipette tips were used to inoculate three TSA plates with 200 μL of homogenized mycelium, and the slurry was evenly spread over the entire surface of a 90-mm plate with

a sterile swab and allowed to dry in a laminar flow hood. The challenging bacterium was then inoculated from a fresh 24-h TSA plate grown at 32°C in a streak down the center of the plate. All assays were prepared in triplicate. Three control plates were prepared as described above by sham-inoculating plates with sterile water. Plates were incubated under ambient conditions and 24-h fluorescent light for 6 days and zones of fungal inhibition recorded. Zones of inhibition were log10(x)-transformed, and a one-way Kruskal–Wallis test or Mann–Whitney *U* test was conducted when appropriate in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) to determine whether antifungal activity differed among congeneric strains of bacteria.

Molecular and Clinical Diagnostics

Before making comparisons between the microbiota of diseased versus healthy snakes, it was necessary to employ multiple diagnostic techniques to determine whether or not snakes were infected with *Oo*. In an attempt to detect *Oo* in skin swab samples, swabs from all snakes were screened for the presence of the fungus using the quantitative real-time PCR (qPCR) assay in Bohuski et al. (2015) and Allender et al. (2015b). Total genomic DNA was extracted from dry swabs using the MoBio PowerFecal Kit and manufacturer protocols. PCRs were run in triplicate on a Roche Lightcycler 480 (Roche Diagnostics Corp., Indianapolis, IN) under the following conditions: 95°C for 3 min, then 50 cycles of 95°C for 10 s and 60°C for 30 s. Each reaction had a total volume of 10 µL and contained 5 µL of IDT PrimeTime Master Mix (Integrated DNA Technologies, Coralville, Iowa), 0.5 µL of IDT PrimeTime qPCR assay (Bohuski et al. 2015), 2.5 µL sterile water, and 2-µL template DNA. Genomic DNA extracted from four pure cultures of *Oo* (#13-42282, 12-34933, 13-40265, 12-33400 isolated in Allender et al. 2015c) and an IDT synthetic gBlocks fragment of the ITS region of rDNA were used as positive controls. Two no-template reactions were run as negative controls to rule out contamination as a contributing factor on all 96-well plates. A sample was considered negative for *Oo* if no exponential phase was observed during the qPCR cycling process.

A single snake with signs of SFD from each host species was selected for further clinical examination, including a rattlesnake collected in Cannon County, TN (specimen TR087), and a racer collected in Dekalb County, TN (specimen BR1). Clinical signs that were observed in the timber rattlesnake included facial swelling, discoloration of scales on the mid-dorsum and ventral mandible (Fig. 1a), erratic muscle movements, and lethargy. Three scale clips, two 4-mm tissue biopsy punches, and two skin swabs were collected from visible scabs and swellings on the head and dorsum. The black racer was lethargic, and gross lesions suggestive of SFD were present, including gray–brown crusty lesions on the rostral and mental scales and retention of the spectacles (Fig. 1b, c). Three skin swabs and one 4-mm tissue biopsy punch were collected from these affected areas. The overall disease state progressed in the racer, and it was eventually euthanized.

Scale clips, punch biopsies, and swabs from both snakes and the carcass of the black racer were submitted to the Southeastern Cooperative Wildlife Disease Study in Athens, GA, for diagnostic testing for the presence of *Oo* and histology. When sufficient tissue was available, the tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, sectioned at 4–5 µm, stained with hematoxylin and eosin, and examined by a veterinary anatomic pathologist via light microscopy. The sections were also stained with periodic acid-Schiff (PAS) reaction or Grocott's methenamine silver (GMS) staining to look for fungal hyphae.

In an attempt to isolate *Oo* in culture, scale clips and swabs from the timber rattlesnake and a piece of retained spectacle taken at necropsy from the black racer were plated on fungal culture plates containing Sabouraud dextrose agar (SDA) and incubated at 30°C. The plates were checked daily, and any colonies matching descriptions of *Oo* morphology (Allender et al. 2015c) were re-plated to obtain a pure culture. DNA was extracted from samples of the fungal cultures using a commercial tissue extraction kit according to manufacturer's directions, and a qPCR assay (described in Allender et al. 2015b) was used for detection of *Oo*, using 1 µL of DNA template and GoTaq® (Promega Corp., Madison, WI). Reactions were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA).

Statistical Analyses

A two-way permuted multivariate analysis of variance (PERMANOVA) was used to determine whether either (1) host species or (2) *Oo* presence had a significant effect on the culturable cutaneous microbiome. The presence or absence of genotyped isolates from the culturable microbiome of all snakes was Jaccard-transformed, and a Bray–

Curtis resemblance matrix was constructed using PRIMER 7 (Quest Research Limited, Auckland, New Zealand). A Type III sum-of-squares PERMANOVA design was used with 999 permutations of residuals under a partial reduced model, and both factors were designated as "fixed." Non-metric multidimensional scaling (NMDS) was used to plot data separately for each factor. Finally, to determine whether a correlation existed between fungal load and the culturable microbiome in snakes that tested positive for *Oo,* fungal load was measured by plotting qPCR exponential amplification curves of swab samples against a standard curve using Roche Lightcycler 480 Software ver. 1.5, and a RELATE (non-parametric Mantel test) analysis was conducted in PRIMER 7. The Jaccard-transformed operational taxonomic units (OTU) matrix and fungal load matrix (square root-transformed data) were compared for this correlation analysis.

Results

Snake Cutaneous Microbiome

We obtained a total of 58 bacterial and five fungal isolates from the 12 host snakes (Supplementary Tables 1 and 2). Host species possessed several microbial associations in common, including strains of *Bacillus flexus,* Family Enterobacteriaceae, and *Stenotrophomonas* spp. (Supplementary Table 1). To our knowledge, this is the first time that *Bacillus flexus* and members of the genera *Curtobacterium, Deinococcus, Erwinia, Neopestalotiopsis, Purpureocillium,* and *Wautersiella* have been isolated from snake skin. All other symbionts have been documented in previous studies and are discussed in depth below.

Challenge Assays

Fifteen bacterial strains, isolated from five different timber rattlesnakes, inhibited *Oo* growth. These antifungal strains belonged to the genera *Aeromonas, Erwinia, Morganella, Myroides,* and *Stenotrophomonas,* and to the families Bacillaceae and Enterobacteriaceae (Fig. 2; Supplementary Table 1). In contrast, only one strain isolated from a black racer inhibited *Oo* growth. This isolate belonged to the Family Enterobacteriaceae. Isolates of *Aeromonas* sp. (Fig. 2d), *Morganella morganii* (Fig. 2e), *Stenotrophomonas* spp., Family Bacillaceae, and Family Enterobacteriaceae (Fig. 2c, h) produced the largest zones of inhibition. All remaining bacterial genera showed no antifungal activity, with the exception of a *Pseudomonas* sp. strain which could not be regrown from cryopreservation and challenged against the pathogen.

Multiple antifungal strains of the genera *Morganella, Myroides,* and *Stenotrophomonas* were isolated. Inhibitory activity of these congeneric strains was compared using Kruskal–Wallis or Mann–Whitney *U* tests, so that potential strain-specific differences could be detected within each genus. Antifungal activity did not differ across four strains in an OTU cluster that included *Morganella* spp. strains (TR087-5.3, TR087-7.4, TR087-7.5, and TR087-7.6; KW statistic = 6.6; $P = 0.0653$). Antifungal strains of *Myroides* spp. (TR087-6.3 and TR087-7.3) also showed no differ-

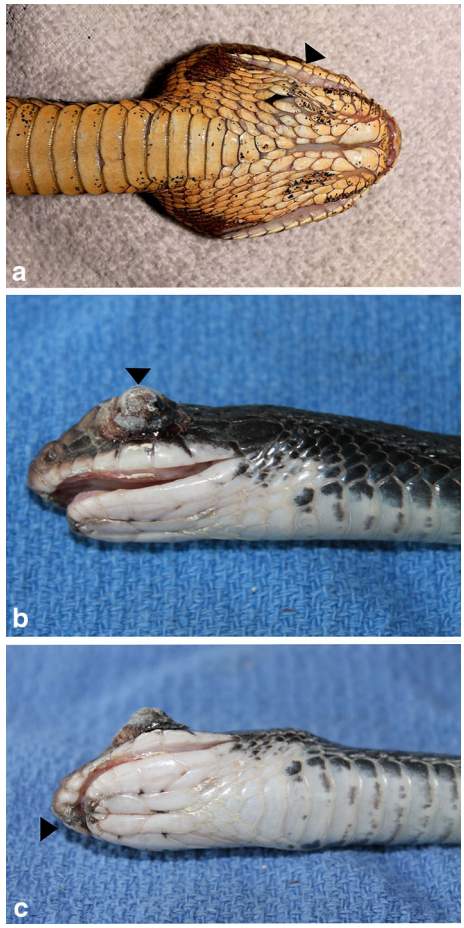

Figure 1. Gross lesions. **(a)** Timber rattlesnake, *Crotalus horridus*, with facial swelling and marked subcutaneous edema (arrowhead). **(b)** Black racer, *Coluber constrictor*. Retained spectacle (arrowhead) is prominent and the periocular scales have a dull gray appearance. **(c)** Black racer, *Coluber constrictor*. Periocular and facial swelling are present, particularly on the rostrum. Gray, dull scales on the ventral mandible correspond to focal regions with sloughed scales and ulceration (arrowhead).

ences in *Oo* inhibition (Mann–Whitney U = 1; $P = 0.3$; Fig. 2). Conversely, differences were observed across strains of *Stenotrophomonas* spp. despite substantial similarity (> 97%) of their DNA sequences. Only three of the six strains assigned to a single reference OTU #1083508 exhibited *Oo* inhibition (Supplementary Table 1). Furthermore, significant differences in antifungal activity were observed across the four antifungal strains of *Stenotrophomonas* spp. (STP759.3, STP780.5, STP1017.1, and STP1017.2; KW statistic = 8.671; $P = 0.0069$).

Molecular and Clinical Diagnostics

Results from qPCR screens of swab samples are displayed in Table 1. Preliminary testing on the scale clips and punch biopsies from BR1 (black racer) and TR087 (timber rattlesnake) did not detect infection with *Oo* by qPCR, fungal culture, or histology. No fungal hyphae were identified with PAS staining in the black racer tissue sample. Ulcerative lesions were present in the oral cavity and the head, but no fungal hyphae were detected in these regions microscopically. Inflammatory lesions were also present in the lung of the racer associated with small numbers of fungal hyphae (images not shown). A tan-colored, powdery fungal colony was isolated from the ocular sample from the racer. Cytology of this colony revealed rectangular arthroconidia, moderate numbers of parallel aleuroconidia, and undulate hyphae consistent with *Oo*. A swab of the fungal colony tested positive for the presence of *Oo* using the qPCR assay described above (Allender et al. 2015b). At necropsy, intralesional fungal hyphae were detected in the retained scales around the eye during histological analyses (Supplementary Fig. 1). Based on the combined results from necropsy and qPCR results for both the skin swab (Table 1) and fungal isolate swab, the authors were able to definitively diagnose SFD in the black racer BR1. From the timber rattlesnake samples (TR087), multiple fungal colonies grew, but swabs of these colonies tested negative for *Oo,* and genotypes of these fungal isolates were not identified. The rattlesnake's skin swab sample also tested negative for *Oo* using qPCR (Table 1), and tissue samples were insufficient for accurate interpretation of histological findings; therefore, a diagnosis of SFD was not confirmed.

Statistical Analyses

No significant interaction was observed between the two factors (species X *Oo* presence/absence; Pseudo-F = 1.3004; P(perm) = 0.146). There were no observed differences in the culturable cutaneous microbiota of timber rattlesnakes versus black racers (Pseudo-F = 1.0299; P(perm) = 0.454), nor were there differences based on the presence or absence of *Oo* (Pseudo-F = 1.3236; P(perm) = 0.155). These results were corroborated by NMDS ordination plots (Fig. 3). Lastly, there was no correlation between fungal load and assemblage of OTUs found on infected snakes (Spearman's $\rho = -0.425$, $P = 0.839$).

Discussion

This study characterizes the culturable cutaneous microbiome of two species of snakes in Tennessee and Arkansas and identifies 16 antifungal species, one of which is a common commensal symbiont of snakes (*Morganella morganii*). We found no effects of host species, presence/absence of *Oo*, or pathogen load on the composition of cutaneous microbiota. Additionally, this study highlights the need for multiple techniques, including molecular, culture-based, and histopathological methods, in order to properly diagnose SFD. We used qPCR, culture-dependent, and culture-independent techniques to confirm infection by *Oo* in a black racer. To our knowledge, this is the first published report of *Oo* causing snake fungal disease in a black racer in Tennessee.

In a timber rattlesnake with lesions, infection by *Oo* was not detected through qPCR, and tissue samples were insufficient for accurate interpretation of histological findings. Variation in the distribution of fungal hyphae and spores along the snake's body can negatively affect detection of *Oo* (Lorch et al. 2015). In addition, the time that this snake was sampled may have occurred outside of the range of effective qPCR detection (day 3 post-infection until day 55; Allender et al. 2015a). We can only speculate that *Oo* caused the lesions, as other microorganisms cannot be ruled out as potential contributors to the overall disease state observed in the rattlesnake. *Fusarium solani*, for example, is known to cause mycotic keratitis in humans (Short et al. 2013), and three strains of this species were obtained from the rattlesnake (TR087-5.1F, TR087-5.2F, TR087-5.3F). However, the significance of the isolation of *F. solani* is difficult to evaluate, because this species is common in the environment and known to rapidly colonize damaged snake skin (Nichols et al. 1999).

Two additional fungal species, *Neopestalotiopsis foedans* and *Purpureocillium lavendulum,* were documented from

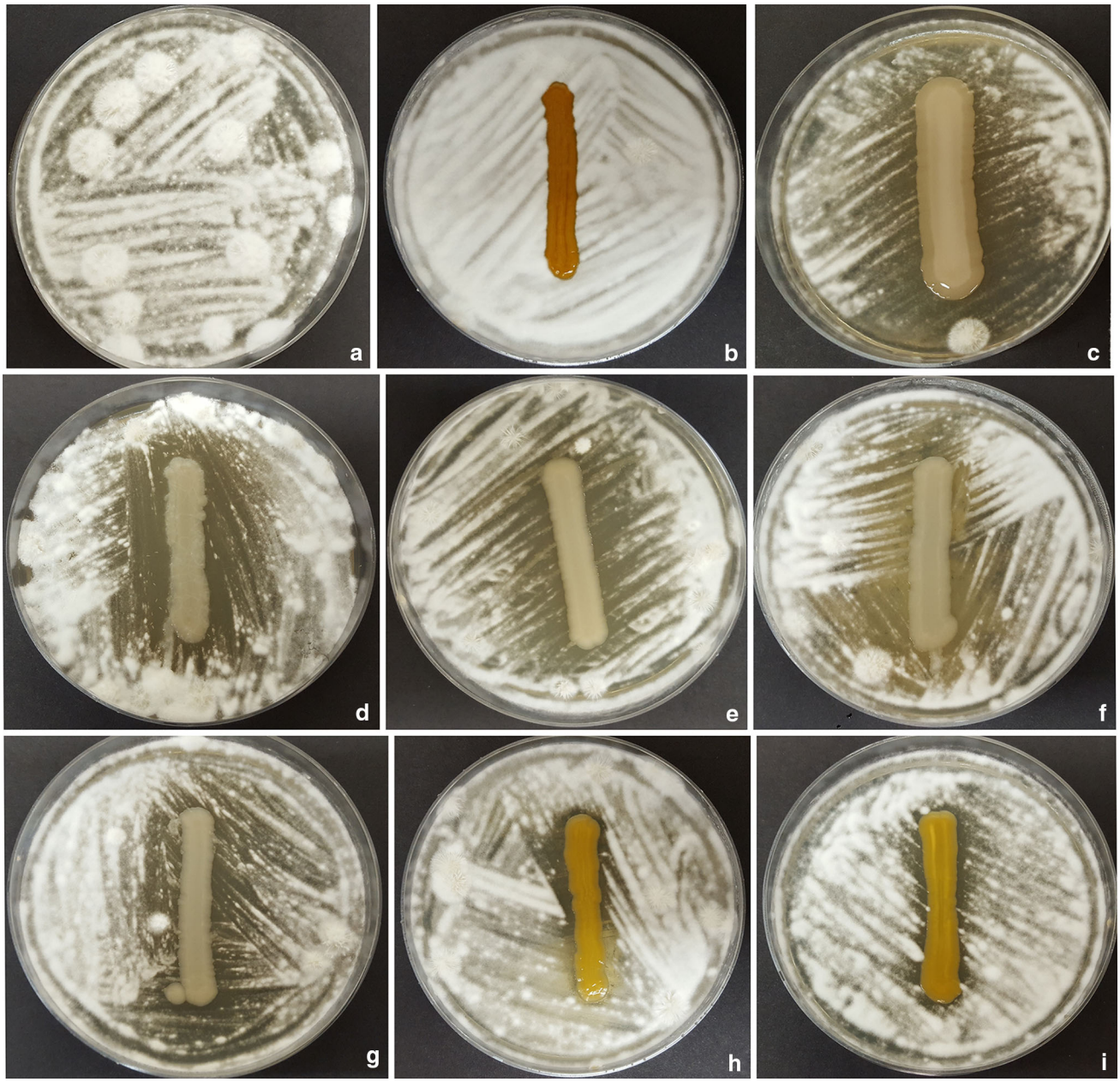

Figure 2. Growth of *Ophidiomyces ophiodiicola* challenged against bacterial isolates in vitro. (**a**) Control plate with a pure lawn of *Oo*. Note that circular colonies formed where large fragments of mycelia were inoculated. (**b**) No growth response of *Oo* to a streak of bacterial isolate BR1.1, *Chryseobacterium* sp. (**c–i**) Growth response of *Oo* to the following bacterial isolates: (**c**) TR087-5.3, Family Enterobacteriaceae sp., (**d**) TR087-5.1, *Aeromonas* sp., (**e**) TR087-7.4, *Morganella morganii*, (**f**) TR087-6.3, *Myroides* sp., (**g**) TR087-7.3, *Myroides* sp., (**h**) TR087-7.5., Family Enterobacteriaceae sp., and (**i**) TR087-7.6, *Morganella* sp.

the black racer that was positively diagnosed with SFD. Species in the genus *Purpureocillium* have been associated with disease conditions in a tortoise and crocodile, but not in squamates (Cheatwood et al. 2003; Perdomo et al. 2013). To our knowledge, this is the first report of *Neopestalotiopsis foedans* associated with snake skin. *Neopestalotiopsis* spp. are common plant pathogens causing strawberry fruit rot and grapevine disease (Ayoubi and Soleimani 2015; Jayawardena et al. 2015).

We documented 18 bacterial genera, most of which have previously been isolated from snakes. Interestingly, multiple enteric symbionts (Family Enterobacteriaceae)

were amenable to isolation from skin swabs during this study, even though the mouth and cloaca were not targeted for sampling (e.g., *Chryseobacterium* sp.; Jho et al. 2011). Other isolations were not surprising. *Morganella, Mycobacterium,* and *Staphylococcus* spp. have all been documented as oral microbiota of healthy snakes (Jho et al. 2011; Reavill and Schmidt 2012) and were hypothesized to play a similar functional role in our sampled snakes. The species *Morganella morganii* has been documented as a commensal member of the snake microbiome in multiple studies (Junior et al. 2009; Jho et al. 2011; Dipineto et al. 2014). We isolated two strains of *Morganella* spp., both with strong anti-*Oo* activity, and conclude that this genus is likely a common, beneficial member of the snake microbiome that may provide protection against invading pathogens like *Oo.* Other isolates, while not inhibitory, are also likely residents. Sheridan et al. (1989) cultured five bacterial genera in common with our study (*Acinetobacter, Bacillus, Citrobacter, Pseudomonas,* and *Staphylococcus*) from the dorsal and ventral skin surfaces of rattlesnakes (*Crotalus atrox*), and we hypothesize that these strains are natural residents of the snake microbiome.

Inhibitory isolates of *Aeromonas, Stenotrophomonas, Erwinia,* and *Myroides* spp. were more difficult to confirm as natural members of the cutaneous microbiome, either because they were isolated from skin around diseased lesions or because they are known to be opportunistically pathogenic (Kado 2006; Looney et al. 2009; Maraki et al. 2012). Isolates of *Stenotrophomonas* spp. were recovered from three diseased snakes during this study, and these species are known to cause infections in other reptiles (Harris and Rogers 2001; Miller et al. 2004). Moreover, Last et al. (2016) isolated *S. maltophilia* and *Aeromonas* spp. from oral tissue of a mud snake (*Farancia abacura)* infected with *Oo.* Species in the genus *Aeromonas* have been isolated from diseased reptiles in other studies (Miller et al. 2004; Schroff et al. 2010), and a single isolate exhibited strong antifungal activity in this study. Collectively, these findings suggest that these inhibitory isolates are likely secondary colonizers of necrotic tissues that may be capable of mounting a defense against *Oo* and other invading pathogens at sites of infection. Alternatively, they may produce a secondary infection that causes more harm to the infected host snake.

Conclusions

In this study, we isolated 58 strains of bacteria from free-ranging snakes belonging to two species, with some individuals presenting clinical signs of SFD. We found that assemblages of culturable cutaneous microbiota did not differ based on host species or infection with the newly emerging fungal pathogen *Oo.* We also found 16 bacterial strains showing inhibitory effects against *Oo*. Fifteen of these are known to be opportunistic pathogens; thus, further investigation is needed to determine whether these isolates serve a defensive or pathogenic role in both dis-

Table 1. Detection of *Ophidiomyces ophiodiicola (Oo)* in Skin Swab Samples from Wild-Caught Black Racers (*Coluber constrictor*) and Timber Rattlesnakes (*Crotalus horridus)* Using Real-Time Quantitative PCR (qPCR).

Specimen #	Species	Location of capture	qPCR detection of *Oo*
BR1	*Coluber constrictor*	Dekalb Co., TN	Positive
STP 761	*Coluber constrictor*	Rutherford Co., TN	Positive
STP 763	*Coluber constrictor*	Cumberland Co., TN	Negative
STP 773	*Coluber constrictor*	Cheatham Co., TN	Negative
STP 633	*Crotalus horridus*	Garland Co., AR	Negative
STP 759	*Crotalus horridus*	Rutherford Co., TN	Positive
STP 779	*Crotalus horridus*	Rutherford Co., TN	Negative
STP 780	*Crotalus horridus*	Rutherford Co., TN	Positive
STP 1015	*Crotalus horridus*	Dekalb Co., TN	Negative
STP 1017	*Crotalus horridus*	Dekalb Co., TN	Positive
STP 1206	*Crotalus horridus*	Cumberland Co., TN	Negative
TR087	*Crotalus horridus*	Cannon County, TN	Negative

All samples were tested in triplicate reactions on a Roche Lightcycler 480 (Roche Diagnostics Corp., Indianapolis, IN). A sample was considered positive for *Oo* if exponential fluorescence was observed during all three trials.

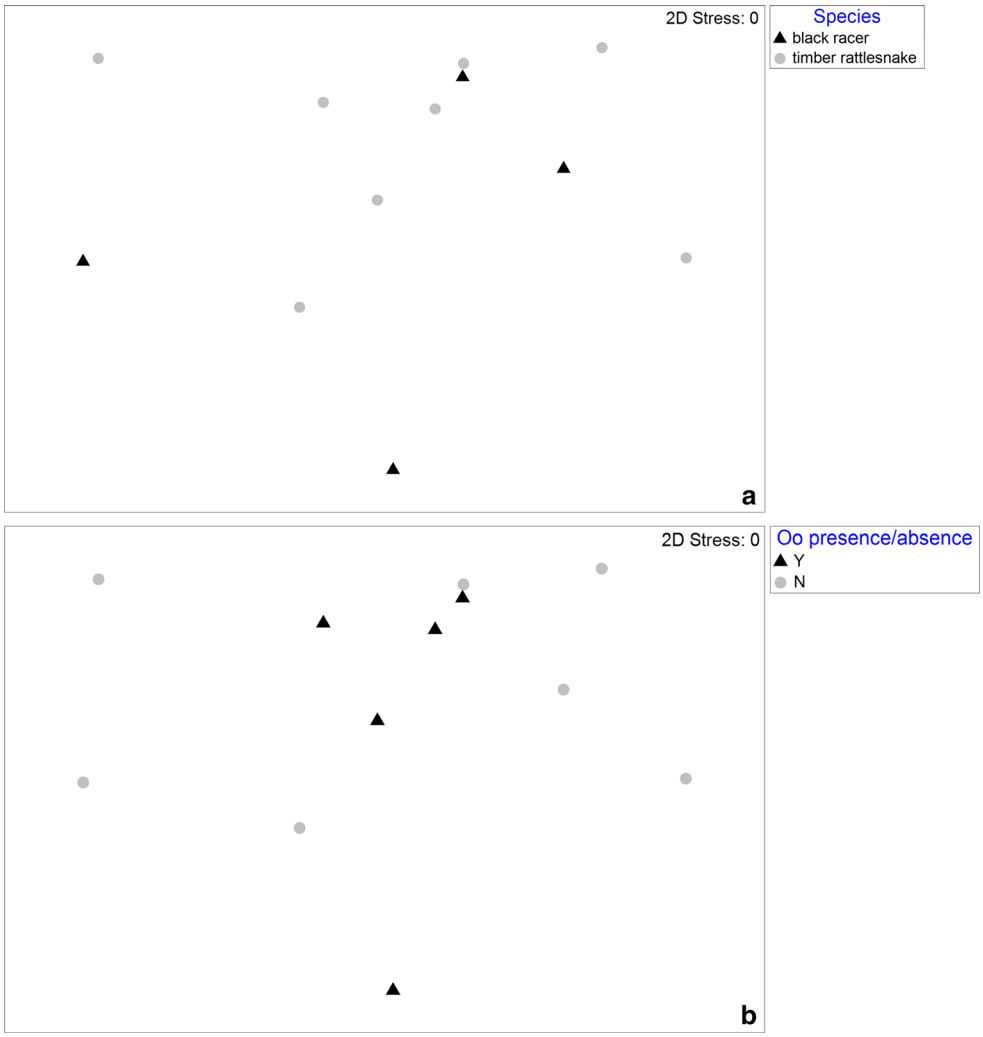

Figure 3. NMDS ordination of Jaccard (presence/absence) transformed OTU data, plotted by a factor of host species (timber rattlesnake or black racer; panel a), and by a factor of *Oo* presence or absence as determined by triplicate qPCR assays (panel b).

eased and healthy snakes. One inhibitory isolate with potent anti-*Oo* defenses, *Morganella morganii,* is a known natural symbiont of snakes. Therefore, we recommend this candidate probiotic species for future therapeutic trials. Additional screening is needed to determine whether *M. morganii* can inhibit fungal pathogens in vivo, while persisting in the presence of cohabiting microbial symbionts without disrupting community structure (Bletz et al. 2013).

Although culture-based techniques are useful for identifying common cutaneous symbionts and evaluating their antifungal activity, the authors acknowledge that these techniques are limited and do not yield comprehensive microbiome data. High-throughput DNA sequencing would provide more detailed snapshots of the resident bacterial community. Such data are important, because community structure and immune function of the microbiome alter disease outcome in the host. Development of an effective probiotic treatment against *Oo* and other dermatophytic fungi will depend on thorough consideration of multiscale ecological factors affecting structure and immune function of the cutaneous microbiome.

Acknowledgements

The authors would like to thank Hannah Carroll, John Arnold, Calvin Hall, Dan Combs, and Vince Cobb for their contributions to field and laboratory work, and the two anonymous reviewers for their helpful comments.

Funding

Funding was provided by the Tennessee Wildlife Resources Agency, Grant 32801-00745, awarded to JEL and DMW, and by the US Fish and Wildlife Service, Multistate Wildlife Grant TN-U2-F14AP00077, Conserving Snake Species of Greatest Conservation Need Threatened by an Emerging Fungal Skin Disease, awarded to RDA.

Author's Contribution

AJH and DMW conceived the experiment, AJH, DMW, MCA, and HF wrote the manuscript, JEL, DB, and RDA captured and sampled snakes, AJH, JEL, GNR, FME, KSM, HF, RDA, and KN collected data and analyzed results, ANM and MCA contributed fungal cultures. All authors contributed equally to the revision process of the manuscript.

Compliance with Ethical Standards

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

Ethical Approval All applicable institutional and/or national guidelines for the care and use of animals were followed (Tennessee Technological University IACUC permit #15-16-001). Sample collection was permitted under TWRA #3886, TDEC #2016-026, and AK #020520155.

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