Microbial Ecology (2021) 82:666–676
https://doi.org/10.1007/s00248-021-01706-x

ENVIRONMENTAL MICROBIOLOGY

Inhibition of *Batrachochytrium dendrobatidis* Infection by Skin Bacterial Communities in Wild Amphibian Populations

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Received: 29 August 2020 / Accepted: 26 January 2021 / Published online: 18 February 2021

Abstract

Skin-associated bacteria are known to inhibit infection by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) in amphibians. It has also been postulated that skin-associated bacterial community is related to *Bd* infection intensity. However, our understanding of host microbial dynamics and their importance in regulating *Bd* intensity is limited. We analyzed *Bd* infection and skin-associated bacteria from two amphibian species, the salamander *Ambystoma rivulare* and the frog *Lithobates spectabilis* that co-occurred in a tropical high-altitude site in central Mexico. Sixty-three percent of sampled salamander individuals and 80% of frog individuals tested positive for *Bd*. Overall, we registered 622 skin-associated bacterial genera, from which 73 are known to have *Bd* inhibitory effects. These inhibitory taxa represented a relative abundance of 50% in relation to total relative bacterial abundance. Our results indicated that, although sharing some bacterial taxa, bacterial community from the skin of both species was different in taxonomic composition and in relative abundance. *Pseudomonas* spp. and *Stenotrophomonas* spp. were among the five most abundant bacterial taxa of both species. Both bacterial taxa inhibit *Bd* infection. We detected that bacterial richness and relative abundance of inhibitory *Bd* bacteria were negatively related to intensity of *Bd* infection independent of species and seasons. Despite the high *Bd* prevalence in both host species, no dead or sick individuals were registered during field surveys. The relatively low levels of *Bd* load apparently do not compromise survival of host species. Therefore, our results suggested that individuals analyzed were able to survive and thrive under a dynamic relation with enzootic infections of *Bd* and their microbiota.

Keyword *Batrachochytrium dendrobatidis*| · Hosts' health · Microbial communities · Skin microbiome · Amphibians · Host resistance

Introduction

All animals and plants harbor microbial communities that frequently have crucial roles in maintaining hosts' health [1]. The skin-associated bacterial communities of amphibians play an important role in host defense against pathogens in synergy with innate and adaptive immune system [2]. Low levels of infection risk in amphibians have been associated with various features of the microbiome, including bacterial species richness [3], specific microbial community assemblages [4], and the presence of microbes that produce metabolites that inhibit pathogens growth [5]. A specific pathogen of global importance is the fungus

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Batrachochytrium dendrobatidis (*Bd*), which has been a major factor in the global decline and extinction of amphibian populations and species [6]. Of 1 240 amphibian species sampled worldwide up to 2013, 516 (42%) were infected with *Bd* [7]. Different amphibian species and populations within species frequently present variations in their vulnerability to *Bd* infection [8].

Although there are multiple factors that might have a role in amphibian resistance to *Bd*, including host genetics, immunology [9], skin peptides [10], and environmental factors [11], bacterial communities living on amphibians' skin also have a crucial role in host's resistance [2]. Several bacteria found on amphibian's skin are known to produce chemical compounds that are able to inhibit growth of *Bd* and to reduce amphibian mortality under laboratory conditions [12]. *Janthinobacterium lividum*, for example, was successfully used to mitigate *Bd* infection in *Rana muscosa* [13], and *Pedobacter cryoconitis* reduced *Bd* load infection in the same frog species [14]. Up to 2015, there were about 255 anti-*Bd* bacterial operational units (OTUs) identified from the skin of 37 species of amphibians that had been deposited in a reference database [15].

Amphibians skin bacteria are not a random assemblage of environmental bacteria [16] maintaining a particular structure across amphibian species, populations, and individuals [17]. Amphibians differ in physical and chemical properties of their skin, producing different suites of antimicrobial peptides [18], mucins and glycoproteins [19], and synthesized alkaloids [20], and according to some authors, these differences can shape the cutaneous bacteria community [21].

The amphibian assemblages of skin bacteria can change seasonally in tropical and temperate zones with marked variations throughout the year in environmental features, such as temperature and precipitation [3, 21]. Adults individuals of *Lithobates yavapaiensis* sampled in winter presented a high infection of *Bd* that coincided with a high OTU richness [3]. Seasonal differences in skin bacteria and *Bd* infection in amphibians are a result of variation in environmental conditions such as temperature, since some host-pathogen responses are temperature-dependent [22]. The optimal immune response of amphibians depends on specific temperature ranges [23], and in cool temperatures, these immune responses could be ineffective [3], leaving amphibians unable to eliminate *Bd*, and some authors have proposed that microbial colonization during winter evolved as a response to fight *Bd* infections [3].

There is an information paucity of how similar skin-associated bacterial communities are among wild co-occurring amphibian species. There is also limited information on the role of specific microbial assemblages in the resistance to *Bd* in wild amphibian populations, as well as on the importance of environmental conditions on the efficiency of anti-*Bd* bacterial skin symbionts. Characterizing these patterns is important to understand evolutionary and ecological processes structuring functionally microbial skin assemblages, which may help in the design of conservation strategies [24].

In the central region of the trans-Mexican volcanic belt, several amphibian species have been reported to have *Bd* infection, including the facultative neotenic salamander *Ambystoma rivulare* and the semi-aquatic frog *Lithobates spectabilis* [25]. Both species occur at the same range but not share habitats [25]. The goal of the present study was to evaluate if two populations of these co-occurring species (*L. spectabilis* and *A. rivulare)* presented different skin bacterial communities and if taxonomic composition and structure of these communities influenced host resistance to *Bd*. Additionally, we evaluated the effect of seasonality on this *Bd* resistance.

Considering that different amphibian species present different levels of susceptibility to *Bd* infection and that skin-associated bacteria have the potential to influence this susceptibility, we hypothesized that skin bacterial composition and structure of *L. spectabilis* and *A. rivulare* were different and that bacterial species richness and relative abundance of inhibitor bacteria were negatively related to *Bd* load, an indicator of the severity of disease symptoms of the host. We also hypothesized that environmental conditions associated with seasons modulate the relationship between bacterial community and *Bd* infection.

Methods

Field Site

Our study was conducted at the core zone of the Monarch Butterfly Biosphere Reserve (MBBR), located in the trans-Mexican volcanic belt on the border of Michoacán state and state of Mexico. The reserve's core zones (13,551 ha) are located above 3000 m and the main vegetation cover consists of fir forests. Climate is humid-cold with a mean annual temperature of 14°C (range = −1.5°C in December to 19.9°C in May) and a mean rainfall of 700 to 1250 mm^3.

We sampled six independent streams (Table 1), each surrounded by fir forest and alpine grasslands in six different periods between 2016 and 2017 (three samplings surveys in

Table 1 Geographic location of the sampled streams in the core zone of the Monarch Butterfly Biosphere Reserve (MBBR)

Streams	N	W
Arroyo Oyamel	19° 37′ 57.38″	100° 19′ 59.73″
Arroyo La Calera	19° 37′ 57.38″	100° 19′ 35. 84″
Arroyo Jesús El Nazareno	19° 37′ 37.55″	100° 19′ 9.86″
Arroyo del Llano El Establo	19° 40′ 24.5″	100°15′59. 6″
Arroyo del Ejido Garatachía	19° 40′ 20.0″	100° 14′ 51″
Arroyo La Mesa	19° 34′ 49.5″	100° 12′ 44.1″

the rainy and three in the dry season). Streams were separated by a distance of at least 1 km. Altitude at the streams ranged from 3000 to 3200 m. *Ambystoma rivulare* was sampled in Arroyo del Ejido Garatachía, Arroyo la Mesa, and Arroyo del Llano el Establo; meanwhile, *L. spectabilis* was sampled in Arroyo Oyamel, Arroyo La Calera, and Arroyo Jesús el Nazareno [25].

Sampling Sites

Along each of the sampled streams, a 100 × 100 m plot was positioned at random. Each transect was 50 m wide from stream banks in a straight line towards uplands. Four people visually searched each transect for 1 hour. Sampling was carried out at daylight (09:00–10:00) and at night (20:00–21:00). To determine the presence of *Bd* and to collect skin bacteria, we took skin samples of *A. rivulare* and *L. spectabilis* according to the methods of Hyatt et al. [26]. All samples are from postmetamorphic adults in the case of *L. spectabilis* and adult individuals in the case of *A. rivulare*. We rinsed each individual with 60 ml of sterile water before swabbing to remove transient microbes, using fresh gloves, and then we took each sample with a sterile rayon-tipped swab, in the ventral and inguinal regions of each collected individual [27]. We standardized the number of swabs per individual and body region and used new gloves to avoid sample cross-contamination [26]. Each swab was placed and stored in vials with alcohol (70%) at −20°C. Before DNA extraction, swabs were dried to evaporate residual ethanol. To prevent *Bd* dispersal, sampling material was disinfected with alcohol (70%) and chlorine bleach (6%), after sampling was completed on each plot.

DNA Extraction

We used swab samples to analyze skin bacterial communities and determine the presence of *Bd* in wild co-occurring populations of the salamander *A. rivulare* and the frog *L. spectabilis*. For DNA extraction of both *Bd* and bacterial communities, we used Prep Man® Ultra (Applied Biosystems). Tubes were centrifuged for 3 min at 13 000×*g*, incubated for 10 min at 100°C. The supernatant was then aspirated from swabs and placed in a sterile 1.5-ml tube. For *Bd* testing, we used Real-Time PCR analysis (qPCR) on an Applied Biosystems Step One Plus TM Real-Time PCR System, following the methodology used in Nava-González et al. [25]. The *Bd* strain used for standards was GPL1 isolated from *Pseudoeurycea leprosa* from the National Park La Malinche, México, and cultured at the Instituto de Biología, UNAM. For PCR reactions and amplification parameters, we used the methods of Boyle, Boyle, and Olsen [28], and each sample was run in triplicate.

To measure *Bd* intensity infection (*Bd* load), we calculated zoospore equivalents (ZE) by multiplying the raw genomic output by 80 as DNA extracts were diluted 80 times through extraction and qPCR [29]. A result was considered positive when the sample amplified (0.1 ZE) before cycle 39 for at least two times; the mean of the two positive samples was used to calculate ZE.

Skin Bacterial Community Metagenomics

Bacterial 16S rRNA amplicon taxonomic fingerprinting was performed amplifying V3 variable region of the 16S rRNA gene with the primer set V3-338f and V3-533r and sequenced in an Illumina MiniSeq at CIAD Mazatlán Unit, Genomic Services, Mexico. Reads were assembled with Flash v.1.2.7 software [30], and VSEARCH [31] was used for further processing, obtaining a relative abundance matrix of bacterial $OTUs_{0.03}$ (Operational Taxonomic Units clustered at 97% identity) that was normalized using the metagenome Seq method [32]. Taxonomic annotation was performed using VSEARCH against RDP database v11.5 [33].

Data Analysis

The *Bd* load and bacterial community were analyzed using two independent and complementary methods. For the first, we used univariate analysis and for the second multivariate analysis. The univariate analysis aimed to evaluate the role of the skin bacterial community in the restraining of *Bd* infection. We used the following parameters, Bacterial Shannon Index (BSI), expected richness (Chao 1), and observed species richness (OSR), that were calculated from the OTUs matrix with the phyloseq package of R software [32]. We normalized the data. The percentage of taxa of inhibitory bacteria (TIB) and the percentage of relative abundance of these taxa (AIB) registered in the two sampled species of amphibians were calculated. Each of the bacteria taxa registered was searched in a reference database of antifungal amphibian skin-associated bacteria [15], and articles published after this review [34–37]. We also reported skin-associated bacteria that have not been tested for *Bd*-inhibitory capacity and bacteria that did not show *Bd*-inhibitory capacity. The number of sampled bacteria taxa listed in the database was used to calculate the percentage of taxa that matched isolates previously shown to inhibit growth of *Bd*. This is a rough estimate of the *Bd*-inhibitory potential of the OTUs observed in this study, and several taxa were previously shown to contain isolates with both *Bd*-inhibitory and *Bd*-facilitating capacities, or no effect on Bd. Thus, the percentage of inhibitory bacteria taxa (IBT) and the percentage of relative abundance of inhibitory bacteria should be considered predictive rather than descriptive measures.

The percentage of this inhibitory taxa relative abundance was also calculated in relation to the total bacterial relative abundance of the samples. To explore the relationship between total bacterial community and *Bd* load in the two

amphibian species (*A. rivulare* and *L. spectabilis*) and the two different seasons (rainy *vs*. dry), we used generalized linear models (GLMs) with an ANCOVA design. We developed one model for each of the parameters of the total bacterial community (observed taxa richness, Chao 1, and Shannon Index) as the continuous explanatory variables and the amphibian species (*A. rivulare* and *L. spectabilis*) and the seasons (dry and rainy) were the categorical variables and *Bd* load was the response variable. We then developed separately a model for the percentage of IBT and the percentage of relative AIB and *Bd* load in the two amphibian species and the two different seasons using also GLMs with an ANCOVA design in which the response variable was *Bd* load and the parameters of inhibitory bacteria were the continuous explanatory variables and the amphibian species and the seasons were the categorical variables. We developed saturated models considering the main effect of the factors and the two and three interactions among the factors. When a term was not significant, the model was reduced to use only significant terms. For GLMs, we used a Poisson error and a logarithmic link function. For each relationship, we obtained a model of the form y = mx + b, where m is the slope and b the ordinate. When significant, differences between slopes were tested among species and season. The regression lines presented in the results were obtained from the GLMs. We conducted GLMs analysis with the R statistical software R 3.6.

The multivariate methods were used to compare the structure composition of bacterial communities in different categories of *Bd* load (ZE= 0, ZE0.01>100, ZE>100) among the two different amphibian species and seasons. For metagenomic data, vegan R library v2.4-6 was used to generate a non-parametric multidimensional scaling plot (NMDS) with $OTUs_{0.03}$ matrix, and a statistical analysis was performed with the ANOSIM function to evaluate groups using the Bray-Curtis distance and 999 permutations.

Results

We sampled a total of 52 individuals, 27 of *A. rivulare* (13 samples from Arroyo del Ejido Garatachía, 13 samples from Arroyo la Mesa, and 1 sample from Arroyo del Llano el Establo) and 25 of *L. spectabilis* (10 samples from Arroyo Oyamel, 8 samples from Arroyo La Calera, and 7 samples from Arroyo Jesús el Nazareno).

Regarding *Bd* infection, we found that 17 (63%) individuals of *A. rivulare* from the 27 sampled were *Bd* positive (95% binomial confidence interval: $p \pm CI = 0.80 \pm 0.15$). From the 25 *L. spectabilis* individuals sampled, we found that 20 (80%) were *Bd* positive (95% binomial confidence interval: $p \pm CI = 0.63 \pm 0.18$). Of these *Bd* positive individuals, 21 were registered in the rainy season (10 *A. rivulare*, 11 *L. spectabilis*), and 16 in the dry season (7 *A. rivulare*, 9 *L. spectabilis*). Overall, we registered 5837 skin-associated bacterial OTUs, 3361 in *A. rivulare*, and 4729 in *L. spectabilis*. Of these bacterial OTUs, 29 phyla, 52 classes, 105 orders, 207 families, and 622 genera were annotated. Of the 52 classes registered, four classes represented about 86 and 96% of the total abundance for *A. rivulare* and *L. spectabilis*. The Class Gammaproteobacteria was the most abundant for both species with ~50% of total abundance (Fig. 1). Of the annotated bacterial OTUs, we registered 516 genera that have not been tested for *Bd*-inhibitory capacity, 33 genera with a non-significant *Bd*-inhibitory capacity, and 73 inhibitory *Bd* bacterial genera, representing on average about 55% of the total relative abundance of bacterial genera (54% in the rainy season and 61% in the dry season for *A. rivulare*; 53% in the rainy season and 57% in the dry season for *L. spectabilis*). Of these identified inhibitory genera, *Pseudomonas*, *Stenotrophomonas*, *Microbacterium*, *Variovorax*, and *Massilia* were the most abundant taxa in both species, *A. rivulare* and *L. spectabilis* (Table 2, Fig. 2). We observed that high bacterial species richness and Chao1 were associated with low *Bd* loads (e.g., ZE = 1.75), while at low species richness, we found *Bd* loads as high as ZE = 853. This negative relationship was significant, and the GLMs indicated no differences in terms of infection prevalence neither between species (*A. rivulare* and *L. spectabilis*) nor seasons (rainy and dry), and the interactions between these factors (Table 3, Fig. 3 a and b). Contrastingly, Shannon index did not explain any of the variations of *Bd* loads. In relation to analysis of *Bd* inhibitory bacteria, we also found a negative relationship in the case of relative abundance (percentage); at high levels of inhibitory bacterial relative abundance, low *Bd* loads were registered. This pattern was independent of amphibian species and season (Table 3, Fig. 3c). Percentage of inhibitory bacterial taxa did not show a significant relationship with *Bd* loads.

Microbial profiles are separated by host species, and for *L. spectabilis*, there was a separation for rain and dry season (Fig. 4). Although ANOSIM values do not stress a significant difference for the 12 analyzed groups (3 *Bd* load categories, 2 host species, 2 seasons), there was a tendency for the bacterial community of *A. rivulare* to group in the rainy season (Fig. 4). We observed a more dispersed community in the dry season for this species. For *L. spectabilis*, a clear group of high *Bd* load in the dry season was observed (Fig. 4).

Discussion

Although the understanding of the dynamics between *Bd* infection and skin microbial ecology in amphibians is rapidly growing [3, 27], the number of field studies on the modulation of this relationship by *Bd* inhibitory bacteria, bacterial community structure, and seasonal effects is still limited. In this study, we address some aspects of these knowledge gaps by

Table 2 Abundances (%) of the genus registered in skin samples of *A. rivulare* and *L. spectabilis* in the Monarch Butterfly Biosphere Reserve during the rainy and dry season

Ambystoma rivulare				*Lithobates spectabilis*			
Genus	Rain		Dry		Rain		Dry
Pseudomonas (In)	33.75	*Pseudomonas* (In)	30.4	*Pseudomonas* (In)	30.35	*Pseudomonas* (In)	37.88
Photobacterium (nt)	11.41	*Stenotrophomonas* (In)	9.35	*Microbacterium* (In)	7.79	*Stenotrophomona* (In)	14.81
Moraxella (nt)	8.78	*Microbacterium* (In)	6.19	*Stenotrophomonas* (In)	7.63	*Variovorax* (In)	4.80
Sulfurospirillum (nt)	6.80	*Limnohabitans* (ns)	4.57	*Devosia* (nt)	3.64	*Microbacterium* (In)	2.79
Actinopolymorpha (nt)	6.67	*Oxalicibacterium* (nt)	3.92	*Ochrobactrum* (In)	2.68	*Devosia* (nt)	2.01
Oceanicaulis (nt)	3.19	*Devosia* (nt)	3.08	*Variovorax* (In)	1.86	*Massilia* (In)	2.00
Tepidisphaera (nt)	2.96	*Ochrobactrum* (In)	3.02	*Luteolibacter* (nt)	1.11	*Ochrobactrum* (In)	1.22
Knoellia (nt)	2.70	*Variovorax* (In)	2.23	*Massilia* (In)	1.06	*Duganella* (In)	1.00
Anaerotruncus (nt)	2.37	*Massilia* (In)	1.46	*Propionibacterium* (nt)	0.63		0.93
Luteibacter (In)	1.76	*Duganella* (In)	0.67	*Duganella* (In)	0.54	*Propionibacterium* (nt)	0.67
Nosocomiicoccus (nt)	1.14	*Roseomonas* (ns)	0.45	*Roseomonas* (ns)	0.47	*Roseomonas* (ns)	0.44
Methyloceanibacter (nt)	0.84	*Propionibacterium* (nt)	0.32	*Acinetobacter* (In)	0.44	*Janthinobacterium* (In)	0.21
Nitrolancea (nt)	0.50	*Rhizobium* (In)	0.28	*Rhizobium* (In)	0.35	*Arthrobacter* (In)	0.19
Portibacter (nt)	0.36	*Salinibacterium* (nt)	0.26	*Arthrobacter* (In)	0.32	*Undibacterium* (ns)	0.19
Oscillibacter (nt)	0.36	*Kaistia* (ns)	0.25	*Advenella* (nt)	0.30	*Simplicispira* (In)	0.18
Enterococcus (ns)	0.36	*Curtobacterium* (In)	0.24	*Pedobacter* (In)	0.28	*Curtobacterium* (In)	0.18
Novosphingobium (In)	0.32	*Leifsonia* (ns)	0.24	*Curtobacterium* (In)	0.26	*Oxalicibacterium* (nt)	0.16
Peptostreptococcus (nt)	0.29	*Arthrobacter* (In)	0.22	*Leifsonia* (ns)	0.26	*Serpens* (nt)	0.15
Neorhizobium (nt)	0.29	*Sphingomonas* (In)	0.20	*Sphingomonas* (In)	0.26	*Luteolibacter* (nt)	0.14
Micrococcus (In)	0.29	*Serpens* (nt)	0.19	*Salinibacterium* (nt)	0.25	*Rhizobium* (In)	0.13
Total	85.1		67.5		60.5		70.10
Number of genus	597		389		447		362

Only the first twenty more abundant bacteria genus, which represent 60–85 % are shown. Below, the total number of genus registered is included. The Bd inhibitory effect is shown (In) for each genus based on Woodhams et al. [15] Assis et al. [34] and Walke et al. [36]. Bacteria genus with a non-inhibitory effect is shown (ns), and bacteria genus that have not been tested for Bd-inhibitory effect is shown (nt)

assessing the relation between structure of skin bacterial communities, number and relative abundance of *Bd* inhibitory bacteria, and seasonal influences on *Bd* infection load of two co-occurring amphibian species. Our results suggest that richness of the skin bacterial community diminishes the intensity of *Bd* infection in both the salamander and the frog species studied. We also found that relative abundance of *Bd* inhibitory bacteria affects the intensity of *Bd* infection. Our results are congruent with studies in similar habitats. Burkart et al. [38] described that a *Bd*-resistant amphibian, *Gastrotheca excubitor*, harbored more isolates of cultivable anti-*Bd* bacteria compared with *G. nebulanastes* with a weaker ability to inhibit *Bd*. Catenazzi et al. [39] assayed the antifungal abilities of 133 bacterial isolates from 26 frog species of the Peruvian Andes, and their results showed that the highest proportion and inhibition strengths of anti-*Bd* isolates were found in two non-susceptible species *G. excubitor* and *Hypsiboas gladiator*.

It is relevant to consider that our results of *Bd* inhibitory bacteria must be considered predictive rather than descriptive since our bacteria database-searching approach was based on taxa and not sequence matching. Besides, according to the literature, the antifungal nature of bacterial species can change

Table 3 Statistics for the GLMs to evaluate the response of Bd load to total bacterial community and the inhibitory bacteria in the skin of wild populations of *Ambystoma rivulare* and *Lithobates spectabilis* in the Monarch Butterfly Reserve, Mexico

Total bacterial community	X^2	*P*
Observed species richness (OSR)	$4.8_{(1)}$	<0.05
Bacteria Shannon Index (BSI)	n.s.	-
Chao 1	$6.6_{(1)}$	< 0.01
Inhibitory bacterial		
Abundance of inhibitory bacteria (AIB)	$9.45_{(1)}$	< 0.001
Species of inhibitory bacteria (SIB)	n.s.	-

In all cases, the effect of species (*Ambystoma* vs. *Lithobates*) and season (dry and rainy) was tested, but not included in the model as they were non-significant (see Fig. 4)

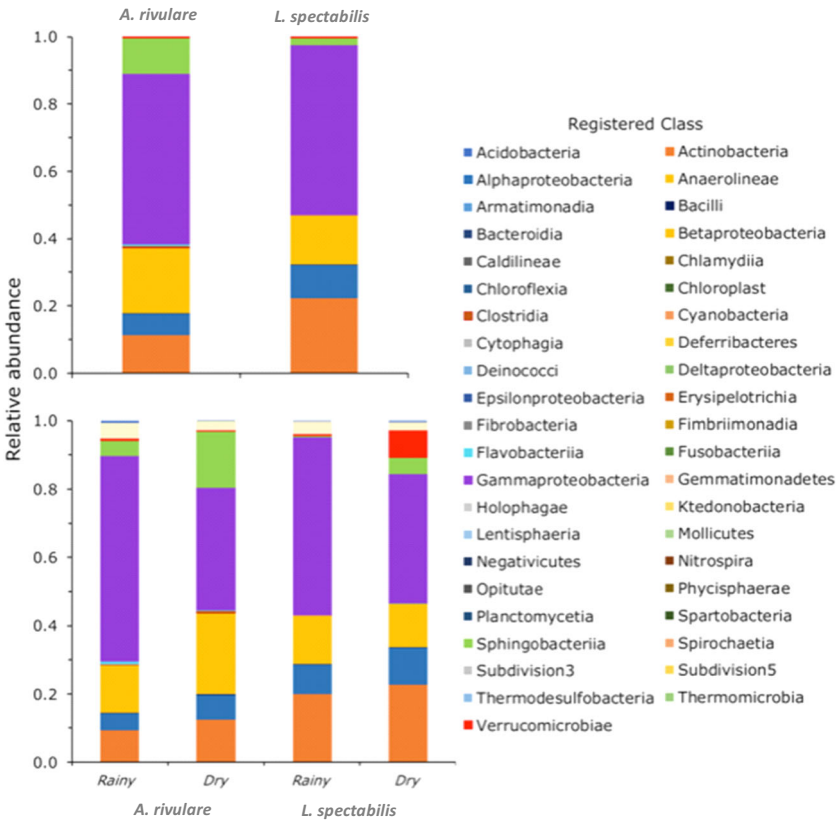

Fig. 1 Relative abundance of taxonomic classes registered in skin samples of *A. rivulare* and *L. spectabilis* in the Monarch Butterfly Biosphere Reserve during the rainy and dry season

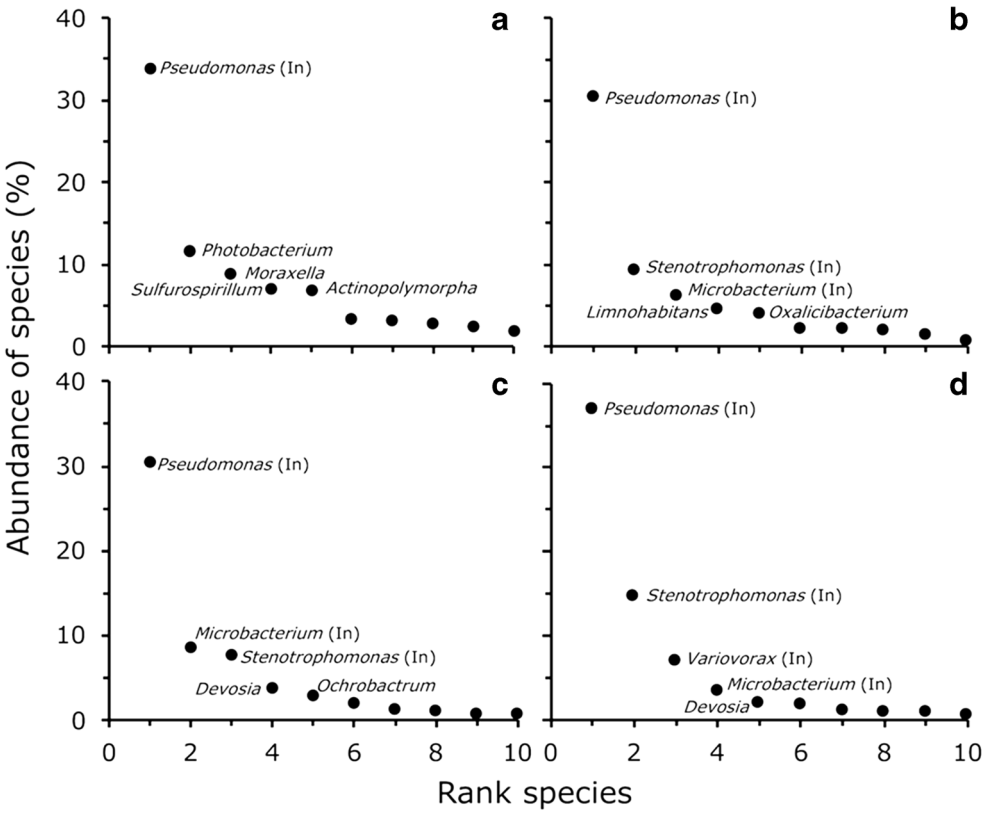

Fig. 2 Rank-abundance curves for the community of genus of bacteria in two amphibian species in the Monarch Butterfly Biosphere Reserve, Mexico. *Ambystoma rivulare* in the upper and *L. spectabilis* in the bottom charts during the rainy season (**a** and **c**) and the dry season (**b** and **d**)

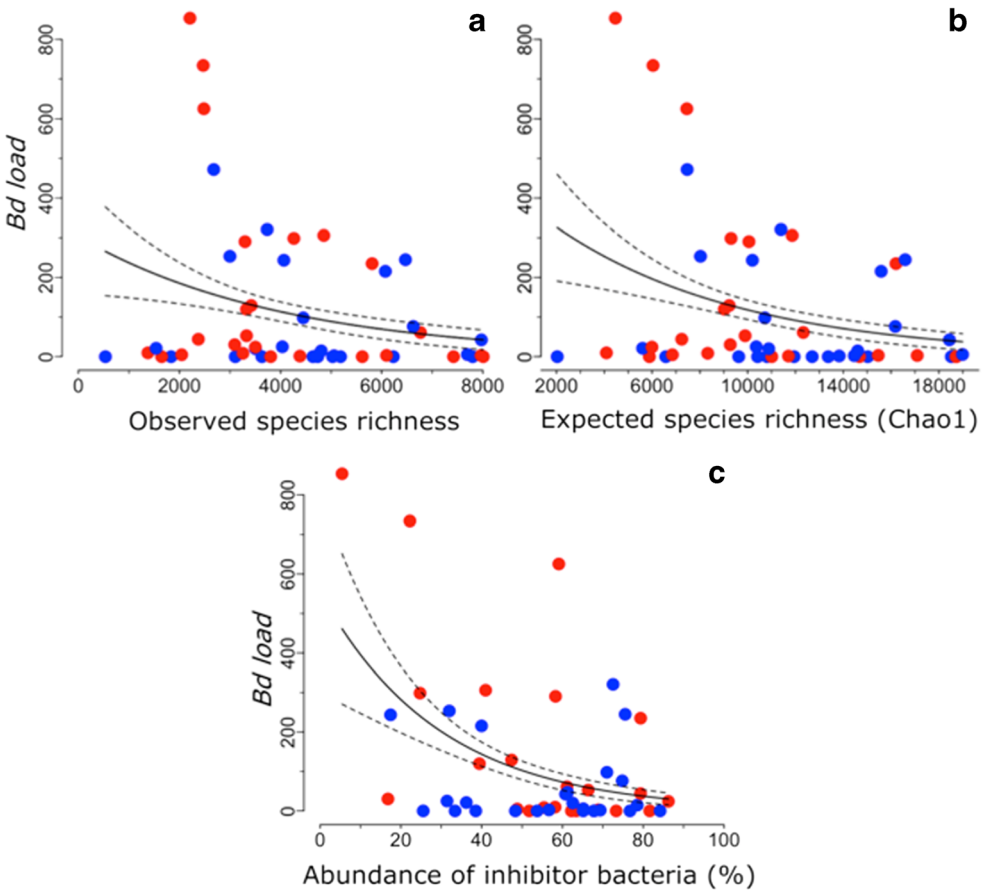

Fig. 3 Relationship among *Bd* load and the bacterial community attributes. **a** Observed species richness. **b** Expected species richness. **c** Abundance of inhibitor bacteria. The trend lines and 0.95 CI were obtained from the GLM Poisson model. We included the effect of species and season; however, these were not different. Species are distinguished in blue for *A. rivulare* and red for *L. spectabilis*, respectively

between in vitro and in vivo conditions [40]. From all published studies about the ability of bacteria to inhibit *Bd*, only 9.2% were experimental trials in vitro, compared with experimental trials on amphibians in vivo that comprised 24.2% of the publications [41]. The OTUs identified in our study could show variation in the *Bd*-inhibition capacity. Besides, most of bacterial OTUs found in this study have not been yet tested for *Bd*-inhibitory capacity.

The number of identified skin-associated bacterial OTUs for each of the studied species, 3361 for *A. rivulare* and 4729 for *L. spectabilis*, falls within the range reported in other amphibian species (e.g., 2667 for *Craugastor fitzingeri* to 10,192

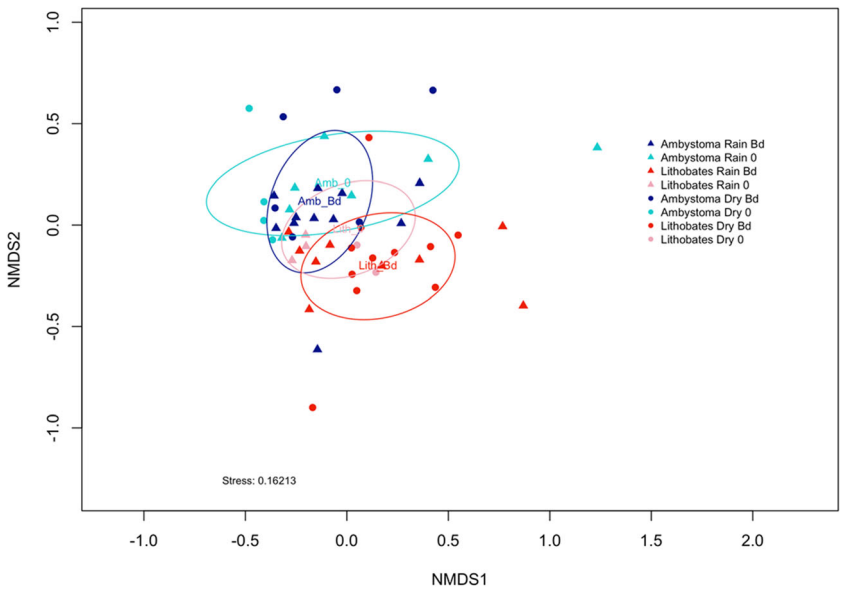

Fig. 4 NMDS analysis of microbial $OTUs_{0.03}$ profiles separated by host species and seasons with a total of 12 analyzed groups (3 *Bd* load categories, 2 host species, 2 seasons). We observe in red colors *L. spectabilis* and in blue colors *Ambystoma rivulare*. In triangles high *Bd* load, in squares low *Bd* load, and in circles no *Bd* load. Microbial community seems to structure depending on both species and season, and *Bd* presence seems to be correlated with certain bacterial community and Bd absence with the dispersion of the bacterial diversity in both amphibian species

for *Alytes obstetricans*) [16, 17, 42], although these variations in reported numbers might be at least partially due to method biases. We registered a significant negative relationship between infection load (number of *Bd* zoospores) and skin bacterial community richness independent of host species and seasons. Several studies have reported negative relationships between skin bacterial community richness and *Bd* load in amphibians [e.g., 3]. Skin bacterial community richness but not diversity was negatively correlated with *Bd* load. Richness can be more sensitive to rare OTUs since all OTUs are counted as either present or absent.

Considering the most abundant bacterial genera for each of the two host species, only four that ranked among the 10 most abundant genera were shared. Additionally, NMDS plots showed little overlap in bacterial community structure, with different dispersion between the two host species. In *L. spectabilis*, the seasonal (rain vs. dry) skin bacterial community structure was also evident in the NMDS plot (Fig. 4). Several studies [16, 17, 42] have also reported differences in composition and structure of the skin-associated bacterial communities within assemblages of amphibian species. Differences in composition and structure of skin microbial communities among amphibian species may be associated with host traits, such as chemical composition of the skin mucus [19], host behavior, skin shedding rate, and diet [42]. Although we cannot be certain, differences in phylogenetic position (host species are included in two distantly related orders, Caudata and Anura) might be the cause, at least partially, of differences in skin bacterial communities between *A. rivulare* and *L. spectabilis*. Rebollar et al. [16] suggest that amphibian species that are phylogenetically closely related might have similar skin chemical conditions, and therefore similarities in bacterial communities.

Additionally, although the studied populations of the two species are co-occurring in the same range, adult stages present differences in life cycle, habitat use, and traits associated with differences in bacterial communities among host species, and such as diet and behavior may contribute to the microbial differences registered in the two species. *Ambystoma rivulare* is an aquatic salamander with a completely aquatic life cycle [25]. This species is associated with slow-flow streams in pine or pine-oak forests and undergoes metamorphosis, but the adults remain in the water [43]. Diet composition in this salamander is markedly dominated by ostracods. On the other side, *L. spectabilis* is a semiaquatic frog with a life cycle associated with water, at least during the mating season during which riparian habitats are used [25]. This frog is rarely seen in purely aquatic habitats, and this species uses rock microhabitats and occasionally pool, sand, plant, river, and anthropogenic substrate microhabitats [44]. Although this species is a dietary generalist that feeds on invertebrates, predation on other anurans has been recorded. Finally, if the environmental bacterial source in both types of habitat is different, it would be reasonable to expect differences in the skin bacterial communities between the two host species.

Notwithstanding differences in skin bacterial communities, the most abundant bacterial genus in the two host species was *Pseudomonas* spp. This bacterial genus is dominant in the skin-associated bacterial communities of several *Bd* non-susceptible amphibian species [16]. The second and third most dominant genus in the skin bacterial community of *L. spectabilis* and *A. rivulare*, respectively, was *Stenotrophomonas* spp. Both *Pseudomonas* and *Stenotrophomonas* genera have been reported to inhibit the intensity of *Bd* infection [31]. At the level of phyla, microbial skin communities in both host species were dominated by the same three phyla (Proteobacteria, Actinobacteria, and Bacteroidetes). The two dominant bacterial genera, *Pseudomonas* and *Streptrophomonas* belong to Proteobacteria, the phylum that dominates skin bacterial communities in amphibians [45] and that comprise about 80% of bacteria with known anti-*Bd* effects [13]. Of the bacterial genera registered, 73 (55% of total bacterial relative abundance) are considered to be inhibitory of the intensity of *Bd* infection [13]. Additionally, *Pseudomonas*, an inhibitory bacterium genus, was the most abundant in both species. Intensity of *Bd* infection measured as *Bd* load ranged for both species between 1.75 and 853 ZE (average ZE = 152 ± 35). Longo et al. [46] consider *Bd* infection to be low in *Eleutherodactylus coqui* when load is less than 100 *Bd* zoozpores, and Jani and Briggs [27] consider a *Bd* load of 10,000 *Bd* cells to be the threshold at which *Rana sierrae* succumbs to chytridiomycosis. Therefore, we can assume that in general, *Bd* infection intensity in both of our studied species ranged from low to moderate.

It is also interesting to highlight the finding of two important bacteria genus in *L. spectabilis*. These bacteria were not always found in amphibian's skin: *Pedobacter* in rainy season and *Janthinobacterium* in dry season. *Pedobacter* species have shown *Bd*-inhibitory effects in *Plethodon cinereus* and *Hemidactylium scutatum* [47]. This bacterium genus is characterized by having a high metabolic diversity, what allows it to be dominant in aerobic communities in aquatic and terrestrial environments, and another important characteristic is that *Pedobacter* thrive over other bacteria genera in changing microclimates [48]. In the case of *Janthinobacterium*, *J. lividum* is recognized as a mutualistic partner with salamanders, such as *Plethodon cinereus* and *Hemidactylium scutatum* [49]. However, *J. lividum* is not present naturally on the skin of many amphibians [47]. In fact, this genus is limited to some habitats. For example, *J. lividum* has been found in many temperate amphibian communities, is extremely rare in low elevation cloud forests [39], and uncommon in tropical regions [39]. This bacterium grows at low temperatures, such as high elevation streams [50] and produces a metabolite, violacein, an agent of chemical defense with broad bioactivity

profile including antibacterial, antiviral, and antitumoral activity, and its application has been proposed as a bioaugmentation or probiotic treatment to control chytridiomycosis [47].

We registered a tendency of higher percentage of relative abundance of *Bd* inhibitory bacteria in the dry season (*A. rivulare*: rainy season 54% vs. 61% dry season; *L. spectabilis*: rainy season 53% vs. 57% dry season). In the dry season, we also registered for both host species a smaller number of *Bd* positive individuals (*A. rivulare* 10 in rainy season vs. 7 in the dry season; *L. spectabilis* 11 in rainy season vs. 9 in dry season). A lower *Bd* load was also registered during the dry season in our study site in the assemblage of amphibians, including the two host species of our study [25]. These findings suggest that environmental conditions such as low temperature (< 7°C) and low humidity (< 50 mm of rain), as well as the higher relative abundance of inhibitory bacteria, might be causal factors in the decrease of *Bd* load in the dry season. Additionally, we also registered a higher bacterial richness in the dry season for *A. rivulare*, but not for *L. spectabilis*. Longo et al. [3] registered a higher skin bacterial richness and higher *Bd* load in *Lithobates yavapaiensis* during the winter. Therefore, our results suggest that there might be a synergic effect of temperature, humidity, bacterial richness, and relative abundance of inhibitory bacteria that results in a lower intensity of *Bd* infection during the dry season.

Although 63 % of sampled individuals of *A. rivulare* and 80 % of *L. spectabilis* were *Bd* positive, no dead or apparently sick individuals were registered during the field surveys. The relatively high percentages of infected individuals of both host species and the low to moderate intensity of *Bd* load level that apparently do not kill or sicken infected individuals suggest that both host species are able to survive and thrive under a dynamic relation with enzootic infections of *Bd*. Relative abundance of *Bd* inhibitory bacterial genera in the skin of both host species might be an important biological defense that limits infection intensity of *Bd* regardless of host species and seasons. Our study sets a precedent in the amphibian's microbial ecology studies in Mexico, and it is also suitable for future comparisons with other similar high-altitude studies to reveal epidemiology dynamics of *Bd* and its relationship with associated bacteria.

Acknowledgements This study was part of the Project 2015-259173/ Consejo Nacional de Ciencia y Tecnología and Project Coordinación de la Investigación Científica-Universidad Michoacana de San Nicolás de Hidalgo-2019. The results of this study are part of the Ph.D. thesis of the principal author, under the direction of ISO. We thank Consejo Nacional de Ciencia y Tecnología for the doctoral fellowship awarded to BANG (CONACyT CVU 444637). Secretaría de Medio Ambiente y Recursos Naturales issued the collecting permits to BANG (SGPA/DGVS710046/15 and SGPA/DGVS710046/16). We thank Jonatan Torres, Hugo Silíceo, Damian Berra, Barush Montejano, Edgar Montejano, Jorge Hipolito, Jerónimo Hernández, Yunuén Soto, Mauricio Guillén, Misael Durán, Aaron Núñez, Vanesa Perez, and José Delgado for their help with the fieldwork.

Availability of Data and Material Sequencing data of this study is publicly available through the NCBI Sequence Read Archive under BioProject PRJNA632638. Pair-end sequences are available for each sample (BioSamples SAMN14916292 to SAMN14916382) through SRA ID SRP261670.

Code availability Not applicable.

Authors' Contribution Nava-González B. and Suazo-Ortuño I. designed the study. Nava-González B. collected the samples. Nava-González B., Raggi L., Gómez-Gil B., Bibian López P., Parra-Olea G., and Maldonado-López Y. contributed in DNA extraction, *Bd* analysis, and skin bacterial community metagenomics analysis. Raggi L. and Gómez-Gil B. contributed with new analytical tools. López P. B., Maldonado-López Y., López-Toledo L., and Suazo-Ortuño I. analyzed the data. Suazo-Ortuño I., Maldonado-López Y., López-Toledo, and Alvarado-Díaz J. wrote the paper. Suazo-Ortuño I. supervised the study.

Funding This study was funded by Project 2015-259173 / Consejo Nacional de Ciencia y Tecnología and Project Coordinación de la Investigación Científica-Universidad Michoacana de San Nicolás de Hidalgo-2019.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflicts of Interest The authors declare no competing interests.

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