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ARTICLE

Microbiota and skin defense peptides may facilitate coexistence of two sympatric Andean frog species with a lethal pathogen

Sandra V. Flechas[1,9] · Alejandro Acosta-González[2] · Laura A. Escobar[3] · Jordan G. Kueneman[4,5] · Zilpa Adriana Sánchez-Quitian[3,6] · Claudia M. Parra-Giraldo[3] · Louise A. Rollins-Smith[7] · Laura K. Reinert[7] · Vance T. Vredenburg[8] · Adolfo Amézquita[1] · Douglas C. Woodhams[4,5]

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Abstract

Management of hyper-virulent generalist pathogens is an emergent global challenge, yet for most disease systems we lack a basic understanding as to why some host species suffer mass mortalities, while others resist epizootics. We studied two sympatric species of frogs from the Colombian Andes, which coexist with the amphibian pathogen *Batrachochytrium dendrobatidis* (Bd), to understand why some species did not succumb to the infection. We found high Bd prevalence in juveniles for both species, yet infection intensities remained low. We also found that bacterial community composition and host defense peptides are specific to amphibian life stages. We detected abundant Bd-inhibitory skin bacteria across life stages and Bd-inhibitory defense peptides post-metamorphosis in both species. Bd-inhibitory bacteria were proportionally more abundant in adults of both species than in earlier developmental stages. We tested for activity of peptides against the skin microbiota and found that in general peptides did not negatively affect bacterial growth and in some instances facilitated growth. Our results suggest that symbiotic bacteria and antimicrobial peptides may be co-selected for, and that together they contribute to the ability of Andean amphibian species to coexist with the global pandemic lineage of Bd.

Introduction

All living organisms establish symbiotic relationships with microbes, and together, they are considered by some authors to be a unit of natural selection [1]. Macroorganisms offer selective microhabitats for the establishment of complex microbial communities. Within these, microorganisms influence the health status of their hosts through interactions ranging from mutualistic to parasitic [2, 3]. Microorganisms play an important role in many processes including food transformation, nutrient uptake, defense against pathogens, and may also be involved in predisposition to different diseases [2, 4–6]. Indeed, symbionts may influence the expression of phenotypes that were traditionally attributed entirely to the host [7, 8]. Consequently, a better understanding of the microbiota and their dynamics, as well as the factors determining the establishment and structure of

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✉ Sandra V. Flechas
vickyflechas@gmail.com

[1] Department of Biological Sciences, Universidad de los Andes, Bogotá 111711, Colombia

[2] Faculty of Engineering, Universidad de la Sabana, Chía, AA 53753, Colombia

[3] Department of Microbiology, Faculty of Sciences, Pontificia Universidad Javeriana, Bogotá AA 56710, Colombia

[4] Biology Department, University of Massachusetts Boston, Boston, MA 02125, USA

[5] Smithsonian Tropical Research Institute, Panama Apartado 0843-03092, Republic of Panama

[6] Environmental Management Group, Department of Biology and Microbiology, Universidad de Boyacá, Tunja 150000003, Colombia

[7] Department of Pathology, Microbiology and Immunology, Vanderbilt University, School of Medicine, Nashville, TN 37232, USA

[8] Department of Biology, San Francisco State University, San Francisco, CA 94132-1722, USA

[9] Present address: Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Bogotá, Colombia

bacterial assemblages could lead to control strategies for a great variety of diseases [9].

Pathogenic fungi are currently recognized as a major threat to animal health [10]. One of the most dramatic examples of population declines is due to the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) [11], which is the causative agent of chytridiomycosis [12]. This disease has strongly impacted amphibian communities [13, 14], causing die-offs and collapses across entire amphibian communities at a scale never before recorded in history [15]. Amphibian species have been affected by Bd in almost every continent [13]. However, despite the catastrophic declines, some species seem to tolerate and may coexist with Bd without displaying clinical signs and are considered asymptomatic carriers [16, 17]. This differential response to Bd infection might be related to host, environment, and pathogen characteristics [18–21].

Symbiotic bacteria and skin defense peptides (often called antimicrobial peptides, AMPs) in the skin mucous layer are among the primary barriers impeding cutaneous colonization by the pathogenic fungus [22–24]. Many bacterial isolates are capable of inhibiting the growth of Bd [19, 25–28]. Moreover, the use of beneficial microorganisms in probiotic therapy via bioaugmentation has been considered one of the most promising strategies for treatment of infected animals and prevention of amphibian population declines caused by Bd [29–31]. In addition, other studies have shown that purified peptides from a wide range of amphibian species inhibit Bd growth under laboratory conditions [32, 33] (reviewed in [34–36]). Anti-Bd abilities differ among peptides (reviewed in [34–36]), and the composition of skin defense peptides in each amphibian species may predict whether they can or cannot resist chytridiomycosis under natural conditions [23] (reviewed in [22]). Despite the large body of evidence supporting the importance of symbiotic bacteria and skin peptides as defense mechanisms against pathogens including Bd, factors determining the structure of bacterial communities remain unclear.

Here, we aimed to understand the microbial component of resistance to Bd infection as well as the role of skin peptides as both a direct defense against Bd and as an indirect defense influencing microbial assemblages. We studied two sympatric species of frogs from the Colombian Andes, the high Andean frog (*Dendropsophus labialis*, Hylidae) and the rocket frog (*Rheobates palmatus*, Aromobatidae). Although, Bd prevalence is high in both species, juveniles appeared to be more infected than tadpoles or adults; however, there is no record of population declines or clinical signs of chytridiomycosis. We hypothesized that differences in Bd prevalence among life stages were associated with skin peptides and skin bacteria. Both species in this study share the same habitat for reproduction, which could potentially mean that both species harbor similar skin microbial assemblages. Thus, we describe the microbial community occurring on the skin of these species, as well as their immediate environment by using 16S rRNA sequencing. We identified the culturable portion of the skin microbiota with antifungal activity and we tested skin defense peptides against Bd to better understand the contribution of each defense in this system of "Bd-tolerant" frogs. Using a database of anti-Bd bacteria, we examined the proportion of bacterial sequences associated with inhibitory OTUs (Operational Taxonomic Units). Lastly, we tested skin peptides to determine if skin secretions are restricting microbial communities, with the hypothesis that evolution may have led to host peptides that support antifungal bacterial communities on the skin of these common Bd-tolerant amphibians. We provide key information on how microbial–pathogen interactions differ across life stages, and how changes in bacterial community composition may affect the response to Bd infection.

Methods

Sample collection

We studied two sympatric species of frogs from the Colombian Andes, *Dendropsophus labialis* (Hylidae) and *Rheobates palmatus* (Aromobatidae). Individuals of both species were sampled in a pond located near Ubaque, Departamento de Cundinamarca, Colombia (04°26′12″ N, 73°55′10″ W, at 1970 m). We visited the site 8 times between 2009 and 2016 (Table 1). We aimed to test whether variations in skin defense peptides and microbiota composition between life stages and species are correlated with Bd prevalence and infection intensity and to determine the role of skin peptides as modulators of the bacterial communities. To address our aims we used a fourfold approach: (1) All animals were swabbed for Bd diagnosis and zoospores quantification, (2) skin swab samples from a subset of individuals and from the environment were used to characterize bacterial communities using targeted amplicon sequencing with Illumina MiSeq, (3) culturable bacteria were identified by mass spectrometry and 16S rRNA sequencing and tested against Bd in order to identify strains with antifungal capacity, and (4) skin defense peptides were tested against Bd and bacteria through growth inhibition assays (see Supplementary Material for additional details).

Batrachochytrium dendrobatidis diagnosis and quantification

For regular PCR, DNA was extracted using GeneReleaser® (Bioventures Inc., Carlsbad, California, USA). The PCR

Table 1 Summary of sampling by species of frog and date

Species	Date of sampling	Sampled individuals	No. of individuals used for:				
			End-point PCR detection	qPCR detection	16S barcode	Culture dependent	Peptide analysis
Dendropsophus labialis	May 2009	23	23	–	–	–	–
	Apr 2010	6	6	–	–	–	–
	Sep 2011	12	12	–	–	–	–
	Jul 2014	18	–	18	18	–	–
	Aug 2014	9	–	9	–	–	–
	Nov 2014	3	–	3	–	–	–
	Apr 2015	10	–	10	–	9	–
	Feb 2016	17	–	16	–	–	17
	Total	*98*	*41*	*56*	*18*	*9*	*17*
Rheobates palmatus	May 2009	43	43	–	–	–	–
	Apr 2010	56	56	–	–	–	–
	Sep 2011	31	31	–	–	–	–
	Jul 2014	26	–	26	26	–	–
	Aug 2014	9	–	9	–	–	–
	Nov 2014	8	–	8	–	–	–
	Apr 2015	16	–	16	–	15	–
	Feb 2016	26	–	20	–	–	26
	Total	*215*	*130*	*79*	*26*	*15*	*26*

Eight field trips were conducted to the same pond in Ubaque, Cundinamarca, Colombia. For each trip, the table provides the collection date and total number of frogs sampled. Each sample was analyzed by one or more of five methods, including diagnostic tests of *Bd* infection by end-point PCR or quantitative PCR, 16S metagenomic survey of bacterial diversity using an Illumina MiSeq, isolation of culturable bacteria, and extraction of antimicrobial peptides. Details are provided in the Methods section

reactions were performed following the protocol described by Annis et al. [37]. For the qPCR Bd assay, DNA was extracted using PrepMan Ultra (Thermo-Fisher Scientific, Waltham, Massachusetts, USA). Extractions and qPCRs were performed following the methods described by Hyatt et al. [38] and Boyle et al. [39]. To quantify infection intensity, we used DNA standards of known concentrations from isolate Bd-GPL (id # CJB57-(4)-p6) and negative controls (see Supplementary Material for additional details).

DNA extraction, amplification, and sequencing of 16S rRNA

DNA for microbial community analysis was extracted from each of the 54 swabs samples (44 from frogs and 10 from water) using the Qiagen DNeasy blood & tissue kit (Valencia, California, USA) following the manufacturer's protocol that was slightly modified. We obtained 16S rRNA gene amplicons using the hypervariable V3–V4 region. PCR reactions were performed in a MJ Research (PTC-200, Hercules, California, USA) and products were visualized by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. We quantified libraries using KAPA Illumina Quantification Kits. PCR products were pooled in equimolar quantities before sequencing on an Illumina MiSeq system (llumina, Inc., San Diego, California, USA; see Supplementary Material for details). Sequence data are deposited in Genbank (BioProject: SRP149982).

Sequence data processing

The 16S rRNA sequences were demultiplexed and quality filtered using quantitative insights into microbial ecology (QIIME) 1.9.1 [40]. Filtered sequences were clustered into operational taxonomic units (OTUs) using the deblur protocol workflow outlined in Amir et al. [41]. Deblur is a greedy deconvolution algorithm that removes low-abundance sequences and resolves related OTUs based on error profiles. This protocol is more conservative than previous approaches in assigning OTU ids, which returns a more accurate estimate of diversity and higher confidence in taxonomic assignments. Using this protocol, we detected a total of 37,167 unique OTUs and a median of 16,423 sequences per sample. We rarefied our dataset to 7,065 sequences per sample to provide sufficient sequence depth within a sample, as well as to retain sufficient numbers of samples for comparison. All samples with sequences abundances below 7,065 were excluded. To explore the

distribution of bacterial taxa that match known Bd-inhibitory taxa, we picked OTUs against the antifungal isolates database [27], using the same OTU picking methodology. First, we trimmed the sequences from anti-Bd isolates in the database to 150 bp. This was then set as the reference database by which to compare our dataset, retaining only 100% matches. Analyses of alpha and beta diversity were conducted in QIIME 1.9.1 and visualizations were generated with R [42] using RStudio. Statistical analyses including LEfSe [43] analysis of relative abundance of microbial taxa among life stages are described below.

Characterization of culturable bacteria

To characterize the culturable bacteria, we followed the isolation procedures described by Flechas et al. [44]. Isolated morphotypes were identified by mass spectrometry with MALDI-TOF (Matrix-assisted laser desorption/ionization time of flight) and Biotyper BRUKER (Billerica, Massachusetts, USA). The profiles were visualized with the software FlexControl (version 3.0) and MALDI Biotyper RTC. For calibration and as a positive control, we used a Bacterial Test Standard (BTS) *Escherichia coli* (DH5α) (Bruker Daltonik GmbH, Bremen, Germany) proteomic profile. Isolates that we failed to identify through mass spectrometry were identified by sequencing the 16S rRNA gene (see Supplementary Materials for additional details).

The antifungal role of the cutaneous symbiotic bacteria

To determine the capabilities of skin bacteria to inhibit Bd, axenic cultures were grown in TGhL broth at 0.5X (5 g tryptone, 2 g gelatin hydrolisate, 1 g lactose, and 1000 mL distilled water) in 15-mL falcon tubes. Cultures were shaken at 250 rpm for 48 h at 25 °C. Samples were centrifuged at 6000 rpm for 5 min at 4 °C. The supernatant was passed through a 0.22-µm syringe filter. We tested each bacterial morphotype against Bd. We set up challenge assays in 96-well microplates (TPP®; Sigma, St. Louis, Missouri, USA), as described previously by Bell et al. [45]. Microplates were incubated at 23 °C during 7 days. Changes in optical density were measured at 490 nm absorbance every 24 h for 7 days using a BIO-RAD 680 (Hercules, California, USA) spectrophotometer.

Effect of antimicrobial peptides on Bd and skin bacterial growth

In order to induce skin peptides release, adult frogs were injected with 40 nmol norepinephrine bitartrate (NE; Sigma, St. Louis, Missouri, USA) per gram body mass. Tadpoles were immersed for 15 min in a solution of 100 µM norepinephrine. Peptides were concentrated, partially purified, and re-suspended following the protocol described by Daum et al. [46]. To accurately determine peptide sample concentration, we used a Micro BCA protein assay kit (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) with bradykinin (Sigma, St. Louis, Missouri, USA) as a standard. Skin defense peptides from each individual (final concentration 100 µg/mL) were tested against Bd strain EV001 [47] in triplicate at a concentration of 2×10^7 zoospores following the methods previously described [35, 46]. Zoospores were harvested using filters of 10 micron pore size (Chemrus, Holliston, Massachusetts, USA).

Identification of antimicrobial peptides in skin secretions

To identify peptide presence in the skin secretions of adults and tadpoles from both species and to confirm the molecular weight of peptides, we used MALDI-TOF mass spectrometry following the protocol described by Holden et al. [48].

Data analysis

To determine if there are differences in Bd prevalence and infection intensity between *D. labialis* and *R. palmatus* and among life stages in each species, we performed a Student's *t* test and a nested ANOVA, respectively. To determine the prevalence of infection, we used the whole dataset including end-point PCR and qPCR results. For infection intensity analyses, we used the subset of samples that were run using qPCR. Since our dataset did not meet the assumptions of normal distribution before running the test, we transformed the data and checked for equal variances using the Shapiro–Wilk test. Venn diagrams were created with the program Venny [49] and were used to visualize: (1) the number of bacterial morphotypes with anti-Bd activity exclusive in each category and shared among life stages of both frog species and (2) the number of OTUs that were shared among life stages and the water. In this case we compared one species at a time.

Measures of alpha diversity for the bacterial community on each individual were calculated using the Shannon diversity index. We used both sets of data, the culturable bacteria and the bacterial 16S barcodes from Illumina MiSeq. To test for significant differences in alpha diversity, we first checked for normality of data within categories and equal variances among categories using the Shapiro–Wilk test. In addition, we inspected the distribution histograms to confirm departures from normality. We calculated Shannon–Weiner diversity index for each individual using the 'diversity' function in the 'vegan' package [50]. We tested for differences in the relative abundance of microbial taxa among life stages of both species with the linear

Table 2 *Batrachochytrium dendrobatidis* prevalence among life stages in two amphibian species

Species	Life stage	Total sampled	*Bd* positives	Prevalence CI (95%)
Dendropsophus labialis	Tadpole	14	0	0 (0–23.1)
	Juvenile	10	4	40 (12.1–73.7)
	Adult	73	30	41 (29.7–53.2)
	Total	*97*	*34*	*35 (25.6–45.4)*
Rheobates palmatus	Tadpole	24	4	16.6 (4.7–37.3)
	Juvenile	94	44	46.8 (36.4–57.3)
	Adult	91	14	15.3 (8.6–24.4)
	Total	*209*	*6*	*29.6 (23.5–36.3)*

Prevalence CI 95% prevalence of infection

In parentheses are the Bayesian credible intervals based on 95% confidence. Bold numbers indicate summaries per species

discriminant analysis (LDA) effect size (LEfSe) method [43]. Weighted Unifrac distance metric was used in beta diversity analyses, and statistical differences between sample types were calculated with Adonis.

The effect of peptides on Bd growth was assessed using a one-sample *t* test. Then to examine the effect of skin peptides on bacterial growth, we calculated the difference between 48 h and 0 h and we compared against the OD values from controls. One-way ANOVA and post hoc Tukey's test were conducted to determine if there was a significant effect of the peptide source on the bacterial growth.

Results

Batrachochytrium dendrobatidis diagnosis and quantification

We procured skin swab samples of 306 individuals, 97 from *Dendropsophus labialis* and 209 from *Rheobates palmatus*, and diagnosed *Bd* infection (Table 1). Ninety-six individuals were diagnosed as positive for Bd (31.3%, CI = 26.2–36.8%) using both diagnostic techniques (qPCR and endpoint PCR; Table 2). We found that Bd prevalence was higher in juveniles, where 46.1% of individuals sampled were positive for the pathogen, compared with 26.8% of adults and 10.5% of tadpoles ($\chi^2_{df=2} = 19.8$, $P < 0.05$). Our results also revealed that infection prevalence was similar in both amphibian species ($\chi^2_{df=1} = 0.66$, $P = 0.416$). Infection intensity (as zoospore equivalents, ZE) ranged from 0.062 to 27,676 ($\overline{X} = 1491$ ZE, SD = 6171) in *D. labialis* and from 0.1 to 1240 ($\overline{X} = 119$ ZE, SD = 322) in *R. palmatus*. The *D. labialis* individual with 27,676 zoospores was an outlier (range without the outlier is 0.062–1422 ZE). The zoospore loads detected here, aside from the outlier, are within the range at which hosts are resisting the infection, rather than tolerating high infection loads that typically cause clinical signs and skin damage in susceptible host species [51]. We did not find differences in zoospore loads either among life stages nested within species (F = 0.5799, $P = 0.4512$) or between species (F = 0.135, $P = 0.714$).

Skin bacterial community composition using bacterial 16S barcode sequencing

After quality control and rarefaction, we used 16 samples from *D. labialis* (adult $n = 8$, juveniles $n = 2$, and tadpole $n = 6$), 21 samples from *R. palmatus* (adult $n = 7$, juvenile $n = 5$, and tadpoles $n = 9$), and nine samples from pond water to examine alpha diversity (or species richness). We found no significant differences in alpha diversity across amphibian species or life stages (Fig. 1). We detected marginal differences in community composition between species when comparing each species, including tadpoles as well as excluding tadpoles (see Supplementary Material). All life stages held significantly lower diversity than the water they inhabited (ANOVA; df = 6, F = 7.924, $P < 0.0001$), and amphibian skin community structure was also different from the microbial community of the pond water (Supplementary Figure 1). Analysis of post-metamorphic host microbial communities (beta diversity) did not indicate strong differences related to Bd infection status (weighted Unifrac Adonis F-$R^2 = 0.07$, $P < 0.137$; unweighted Unifrac Adonis F-$R^2 = 0.083$, $P < 0.001$). Furthermore, FDR-corrected group significance test did not identify bacterial OTUs that strongly distinguish infected individuals from the uninfected, however, two OTUs in the genus *Polynucleobacter* (Oxalobacteraceae) had means of several hundred sequences detected on uninfected individuals compared to zero sequences detected on infected individuals. Analysis of host microbial communities did show significant differences within species by host life stage (unweighted Unifrac Adonis F-$R^2 = 0.26$, $P < 0.001$ for *R. palmatus* and Adonis F-$R^2 = 0.285$, $P < 0.001$ for *D. labialis*; Fig. 2), but only *R. palmatus* displayed a

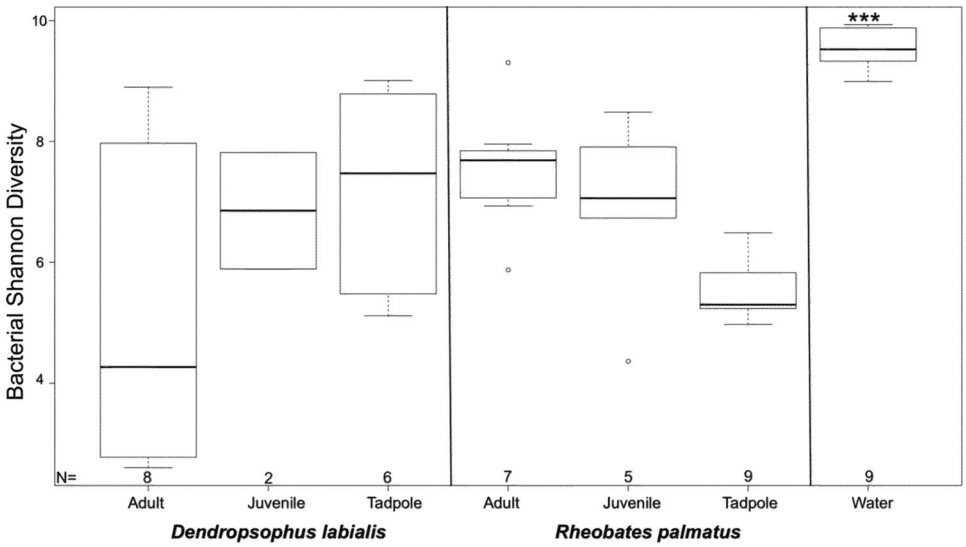

Fig. 1 Alpha diversity (species richness) of bacterial communities in each species, life stage, and pond water. The asterisk indicates significant differences between the water and amphibian hosts

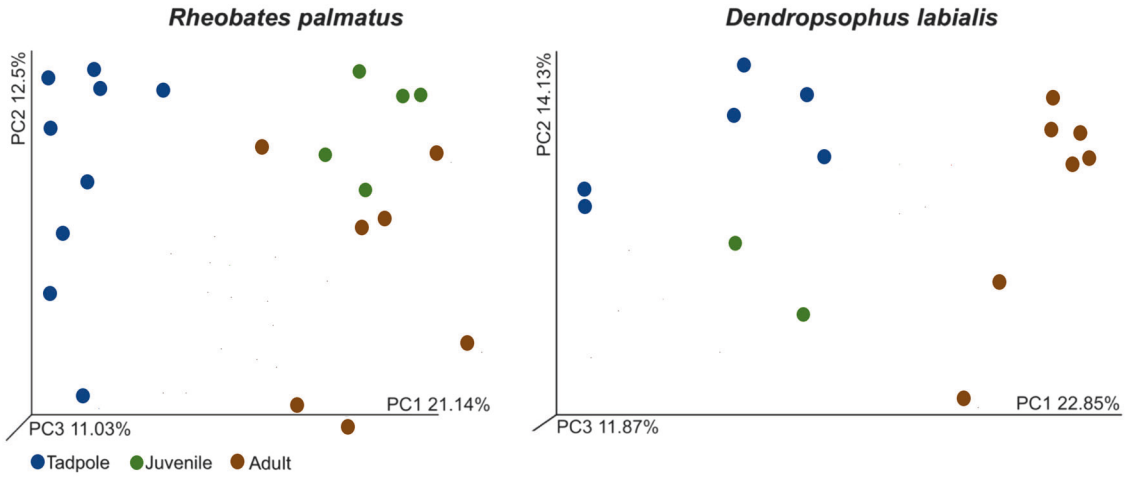

Fig. 2 Principal coordinate analysis of beta diversity of skin microbial communities for each amphibian species differentiated by life stage. Each point represents the skin bacterial community of an individual frog. Symbol and color indicate the life stage and species. Weighted Unifrac distance metric was used in beta diversity analyses

significant difference in the composition as compared using weighted Unifrac. Life stage differences are seen in *R. palmatus* (weighted Unifrac Adonis F-$R^2 = 0.379$, $P < 0.001$). Differences in weighted Unifrac were not significant for *D. labialis* (weighted Unifrac Adonis F-$R^2 = 0.195$, $P < 0.13$). All life stages in both species were dominated by the class Betaproteobacteria, and host life stages differed in their proportional abundance of many other taxa. In particular, in *R. palmatus* Gammaproteobacteria were present in juveniles (5.3%) and adults (11.4%) and almost absent in tadpoles (0.1%) (Fig. 3, Supplementary Figure 2). According to the LEfSe analyses, Flavobacteriia increased in abundance from tadpoles (6.4%) to adults (39.3%) in *D. labialis* (Supplementary Figure 3, Table S1). Bacteria that match isolates known to inhibit Bd according to the public database [27] were found to be unequally distributed across life stages of each species (*R. palmatus* = ANOVA; df = 2, F = 4.527, $P < 0.0255$ and *D. labialis* ANOVA; df = 2, F = 10.58, $P < 0.00188$). Our data show that the highest sequence abundance of putatively anti-Bd bacteria occurred in adults of *D. labialis* and then secondarily in adults of *R. palmatus*. The bacterial taxa that were proportionally most abundant were in classes Flavobacteriia and Betaproteobacteria (Supplementary Figure 4). For both amphibian species, we found five OTUs with more than ten sequences present on tadpoles and also on adults. Four out of five of the OTUs were shared by the two species and one is uniquely present in each species. For *R. palmatus,* the unique OTU was in the family Methylophilaceae, and for *D. labialis*, was in the family Xanthomonadaceae.

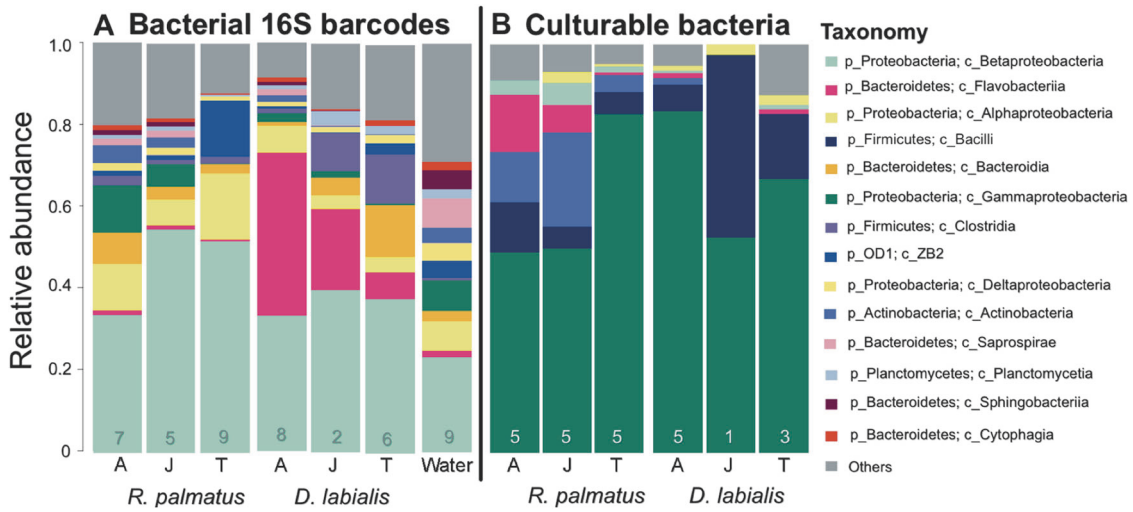

Fig. 3 Relative abundance of bacterial phyla by species and life stage. **a** Relative abundance of bacterial 16S sequences obtained through 16S sequencing of microbiota in two amphibian hosts across life stages and in pond water samples. **b** Relative abundance of isolates recovered through culturable-dependent techniques. The letters along the bottom indicate life stage, A—adult, J—juvenile, T—tadpole. Numbers inside the bars denoted the number of samples per category

Identification of culturable bacteria and their ability to inhibit Bd growth

We obtained 615 bacterial isolates, 468 from R2A agar and 147 from oatmeal agar. Using MALDI-TOF, we identified 548 bacterial morphotypes at least until the genus level. Based on a dendrogram calculated using the Biotyper software, we chose at least two representative isolates per cluster that were selected to confirm the identity by sequencing of 16S rRNA gene. The 67 isolates that were not identified by MALDI were confirmed by 16S rRNA sequencing. In total we identified 100 bacterial morphotypes belonging to 36 genera and four phyla (Supplementary Figure 5). We recovered the highest culturable fraction of isolates in tadpoles of *R. palmatus* (58%) and adults of *D. labialis* (57%) (Table S2). Using the data obtained through culture-dependent methods, we found that *R. palmatus* harbors a higher number of morphotypes per class compared with *D. labialis* ($t = 2.278$, $df = 17.9$, $P = 0.03$; Fig. 3), but we did not detect significant differences among life stages within species (for *R. palmatus*: $F = 3.065$, $P = 0.08$, for *D. labialis:* $F = 0.367$, $P = 0.7$). Members of the classes Gammaproteobacteria and Bacilli were common in both amphibian species. Bacteria in the classes Actinobacteria and Flavobacteriia were most abundant in adults and juveniles of *R. palmatus* (Fig. 3). Species from the genus *Pseudomonas*, *Enterobacter*, *Bacillus,* and *Raoultella* were highly represented with 41.4%, 12.3%, 9.0%, and 3.1%, respectively (Supplementary Figure 5). The number of bacteria with anti-Bd properties that were shared among life stages was very low (Supplementary Figure 6). We identified three species including *Bacillus subtillis, Chryseobacterium joostei,* and *Pseudomonas chlororaphis* present in tadpoles, juveniles, and adults from *R. palmatus*. In the case of *D. labialis*, the only bacterial species shared among the three stages was *B. cereus*. *Pseudomonas koreensis* is the only bacterium that was present in both amphibian species and in all life stages.

To determine anti-Bd activity and differences in bacterial performance among amphibian species and life stages, we challenged the isolated bacteria against Bd. Given the redundancy in the isolated bacteria, we chose one bacterial isolate per life stage in each species for each cluster determined by MALDI-TOF, and therefore we only tested 25.8% of the isolates (159 from 615). We found evidence of antifungal activity in 126 (80%) of the isolates, which correspond to 72 out of 100 identified species. We detected the anti-Bd activity in members of 27 genera in *R. palmatus* and 16 genera in *D. labialis*. Across life stages, the highest number of isolates with anti-Bd activity was found in tadpoles from *R. palmatus* with 31 isolates, followed by 21 in adults and 15 in juveniles. For *D. labialis,* we detected 21 anti-Bd bacteria in adults, 12 in tadpoles, and eight in juveniles. In addition, we found that some isolates facilitated Bd growth, including species from three genera for *D. labialis* and ten for *R. palmatus* (Table S3).

Effect of antimicrobial peptides on the skin bacterial community composition

Peptides were sampled from 30 tadpoles (in groups of five individuals for a total of six samples) and 20 adults of *R. palmatus*, and three tadpoles (one sample) and 16 adults of *D. labialis*. We found that both amphibian species secrete

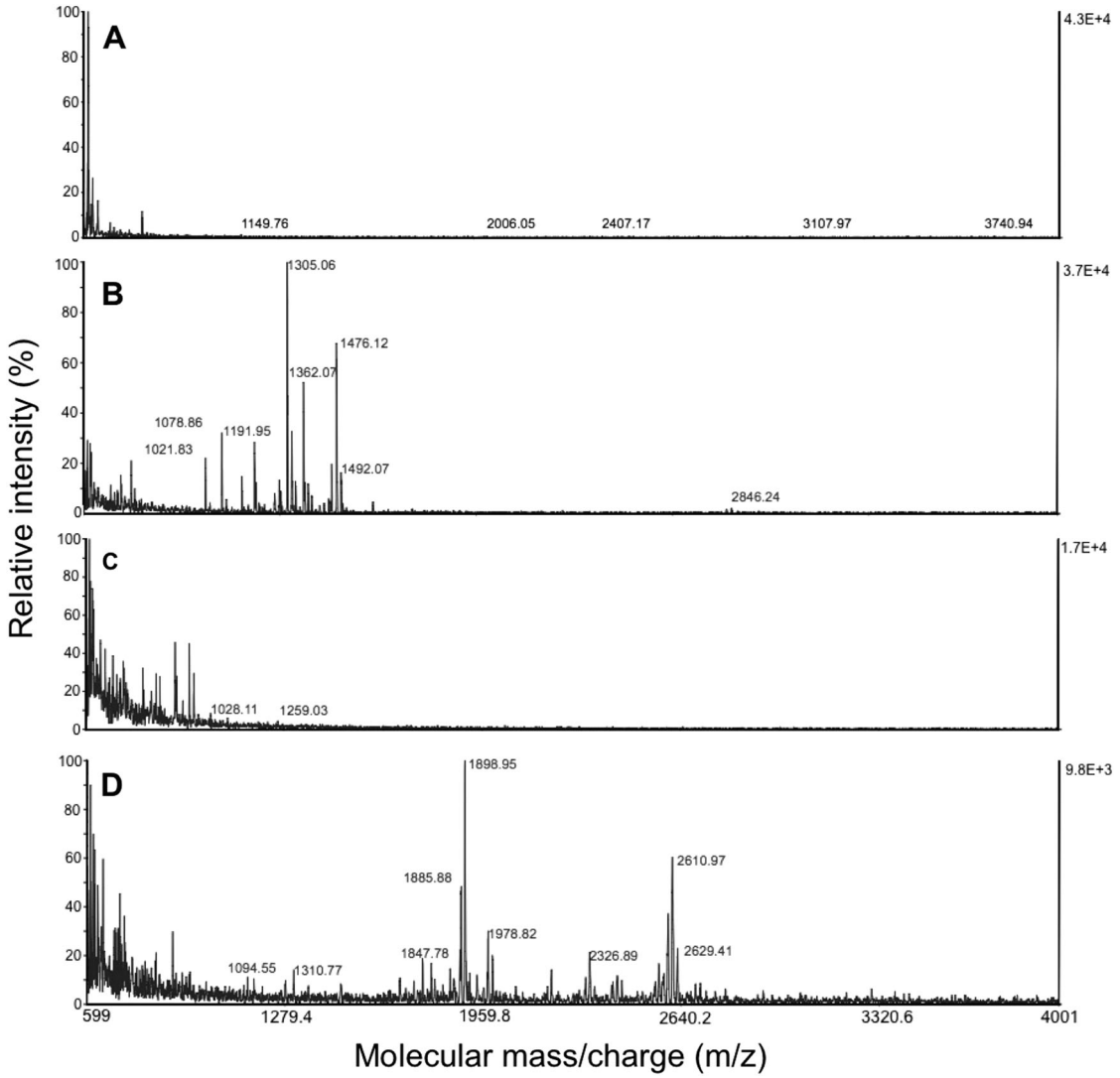

Fig. 4 MALDI-TOF mass spectrometry profiles of skin peptides. **a** *Dendropsophus labialis*——tadpole, **b** *Dendropsophus labialis*—adult, **c** *Rheobates palmatus*—tadpole, and **d** *Rheobates palmatus*—adult. Note that the noise in (**a**) and (**c**) (spectra from tadpole samples) indicate a lack of peptide signals

skin peptides that can inhibit Bd. Total quantities of peptides collected per species/life stage varied between 41.9 and 1918.6 µg (Table S4). The species have different peptide profiles and do not express adult-type peptides as tadpoles (Fig. 4). Adult peptide profiles showed marked differences between species (Fig. 4). In *D. labialis*, we identified a number of peaks with mass signals between 1000 and 2000 (mass to charge ratio, *m/z*). In *R. palmatus* two peaks were consistently found in all samples, the first one at 1898.96 *m/z* and the second peak at 2610.98 *m/z*. We found that most of the natural peptide mixtures inhibited Bd growth at the concentration tested (100 µg/mL; $t = -4.0062$, $df = 34$, $P = 0.0002$; Fig. 5). Our results indicate that skin defense peptides in general do not inhibit host bacterial growth at the relatively low concentration tested here, and growth of some bacteria was facilitated by the peptides (Fig. 6). In addition, the effect of skin peptides varies with the source of the peptides and with the origin of the bacteria (Supplementary Figure 7).

Discussion

Ontogenetic changes in susceptibility have been demonstrated to occur in a number of amphibian species, in which recently metamorphosed individuals are facing a greater risk of disease due to higher infection intensities and lower immune function compared with adults [52, 53] (reviewed in [54]). For many species, the response of the host varies significantly depending on the life stage, suggesting that the development of the immune system including antimicrobial peptides has a key role in preventing morbidity and

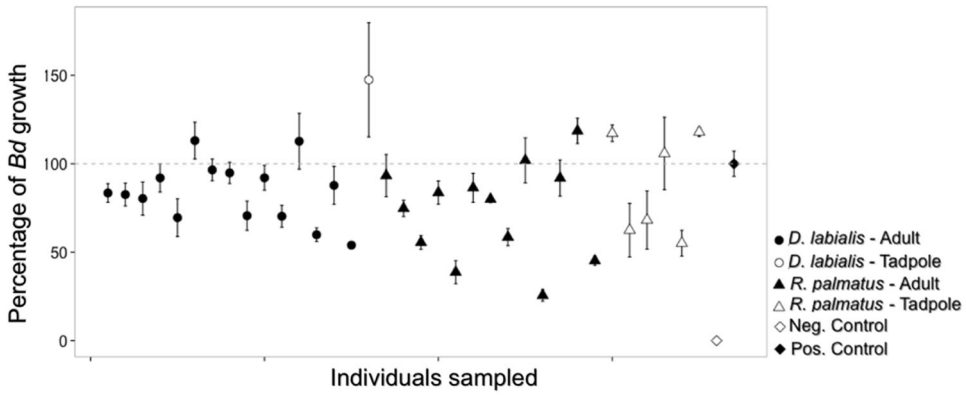

Fig. 5 Variation among frogs in the effect of their skin antimicrobial peptides on Bd growth. Peptide samples were collected from *Dendropsophus labialis* adults (black circles, $N = 15$), *D. labialis* tadpoles (white circles, $N = 1$), *Rheobates palmatus* adults (black triangles, $N = 13$), *R. palmatus* tadpoles (white triangles, $N = 6$). Vertical bars indicated ± 1 standard error. Positive control (black diamond) and negative control (white diamond) are included. The dotted line represents the growth of Bd in the absence of peptides

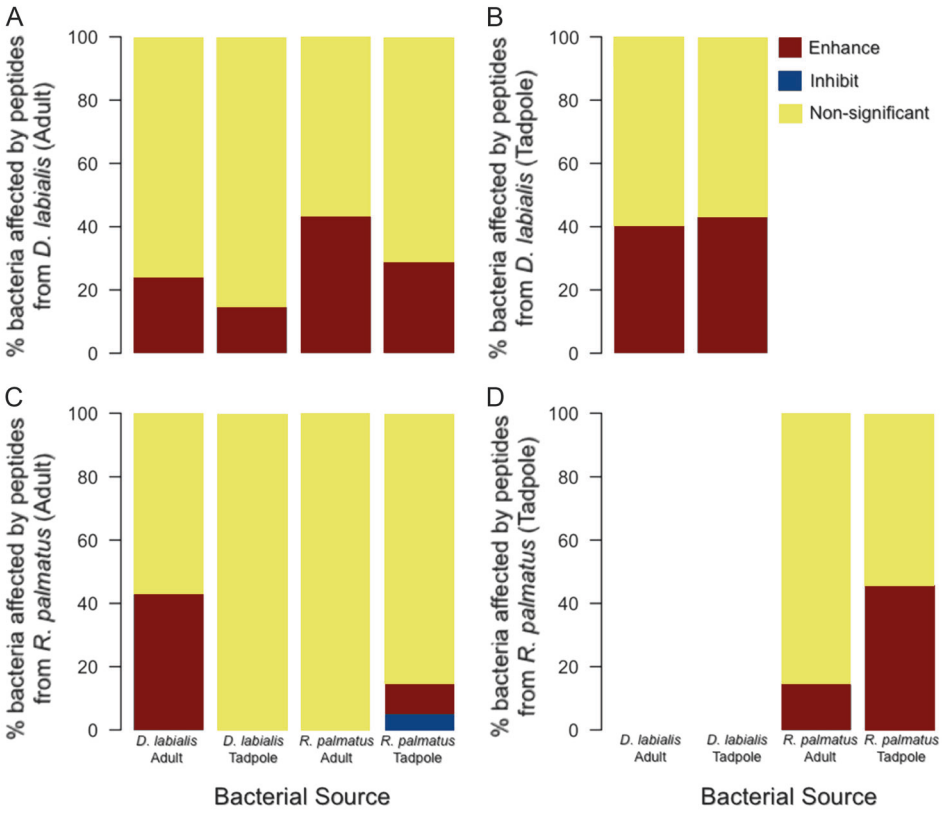

Fig. 6 Effect of antimicrobial peptides on bacterial growth. Each panel shows the proportion of affected bacteria when exposed to peptides from four different sources (adults and juveniles of *Dendropsophus labialis* and *Rheobates palmatus*). Bars represent the source of bacteria. Light gray represents the proportion of bacteria that were not significantly affected by peptides. Dark gray represents the proportion of bacteria whose growth was significantly enhanced by peptides. Black represents the proportion of bacteria that were significantly inhibited by peptides. White spaces indicate that this test was not performed

mortality [48, 55]. Indeed, our study shows that skin peptide defenses are not present in tadpoles of these species, and a delay in peptide expression after metamorphosis may increase infection risk on recent metamorphs. Developmental changes in skin physiology are likely linked to changes in skin microbiota. Here, and in recent studies [56, 57], pronounced ontogenetic shifts in bacterial community composition follow metamorphosis. Thus, changes in bacterial assemblages and the reorganization of the immune system during the transition from tadpole to juvenile may facilitate skin colonization by Bd. This might be due to the potential lack of peptide defenses as previously demonstrated for other amphibian species [48, 58]. Here we reported that the composition of the microbial communities is mainly defined by the life stage, and a lower proportion of Bd-inhibitory isolates in juveniles as well as a potential reduction of peptide defenses might explain a higher prevalence in this stage compared with tadpoles and adults.

Several bacterial morphotypes have demonstrated excellent capabilities of reducing the severity of chytridiomycosis or pathogen growth in vitro [19, 44]. Thus, the presence of bacterial species with antifungal properties is considered one critical factor conferring resistance to Bd. We hypothesized that *D. labialis* and *R. palmatus* could have higher proportions of microorganisms with antifungal properties or a few microbial species with outstanding anti-Bd capacities, compared with susceptible species. Approximately 86% of the tested isolates in *D. labialis* and 76% in *R. palmatus* inhibited the fungus. The high proportion of culturable bacteria exhibiting anti-Bd properties in this population may be an indication of their importance in host protection, as suggested by Becker et al. [28] for Panamanian amphibians persisting in areas with Bd, where 75% of the isolates tested exhibited some ability to inhibit Bd in vitro. Our results are consistent with the idea that symbiotic skin microbes harbored by these two species of Andean frogs are allowing them to cope with the pathogen. In other Andean frogs, differences in susceptibility to Bd were also associated with proportions of beneficial bacteria [59]. However, it is important to consider that anti-Bd capacity may differ when the bacterium is present in a microbial community on frog skin compared to growth in isolation on standard medium and constant temperature.

Our results, along with other studies, suggest that the range of microbial species with antifungal potential is wider than previously considered [3]. Since various studies have found that some bacterial morphotypes from specific groups (e.g., *Pseudomonas*, *Serratia*) are effective inhibitors of the fungal pathogen [27, 60], further research is necessary to determine if these new candidates meet the requirements to be used as probiotics. It is also key to determine the inhibition spectrum of symbiotic bacteria because antifungal capacities may differ among Bd strains [61, 62]. Moreover, variations in the degree of inhibition among bacterial strains belonging to the same species must be examined, since different strains could have different effects on Bd growth [44]. In addition, the anti-Bd function may differ on hosts or in different life stages or microbial communities. This was the case with *Janthinobacterium lividum*, which has been successfully used in the field to increase survival of the threatened mountain yellow-legged frog in the Sierra Nevada [63]; however, it was not effective on the tropical frog *Atelopus zeteki*, failing to persist in the skin and to prevent mortality [64]. This demonstrates the urgent need to find local solutions to fight the pathogen. In our study system *J. lividum* was very rare, with only one isolate out of 615 morphotypes recovered by culture-dependent techniques and only 0.02% of the OTUs using Illumina.

Several studies have evaluated the association of Bd-related declines and the synthesis of skin defense peptides [23, 24, 65]. Along with symbiotic bacteria, we found that skin defense peptides secreted by the granular glands may contribute to disease resistance. Despite the effectiveness of some peptides killing the fungal pathogen, and the capacity to regulate the growth of different microbes, studies that experimentally evaluate the role of skin defense peptides in structuring microbial communities are lacking. Because we detected differences in the culturable skin-associated bacteria among life stages, we hypothesized that the observed variation might be explained by the differential effect of skin defense peptides on bacteria, either promoting the growth of their own microbiota or impeding colonization from non-native bacteria. For this, we tested the effect of antimicrobial peptides on the growth of bacteria in two ways: inter-species and inter-life stages. Skin defense peptides in this system tended to enhance growth of bacteria found in both amphibian species (Supplementary Figure 7) and only rarely to inhibit it (Fig. 6c) at the concentration tested, while low concentrations of skin defense peptides inhibited Bd growth (Fig. 5). Environmental isolates were not cultured in this study, but one expectation is that the amphibian defense peptides would be more effective at inhibiting growth of microbes that do not tend to colonize the amphibian skin. We hypothesized that the beneficial bacteria in our system may have evolved to survive on host amphibians that share the same environment and thus, resist or even utilize host skin peptides. Although not tested in this study, we suggest that environmental bacteria that could harm or not benefit the amphibian host would be more likely to be inhibited by skin defense peptides. Indeed, *Curtobacterium albidum* isolated from tadpoles of *R. palmatus* was inhibited by skin peptides and did not display anti-Bd capacity, and might represent a potential pathogen [66]. Although we mostly detected a positive effect of peptides on bacterial growth, additional factors not evaluated in this study (e.g., physiological differences between larvae and adult) might interact with skin defense peptides to determine which microbes can colonize and persist in the skin, leading to the differences observed among life stages.

We found high prevalence of infection in both amphibian species, with around 30% of the individuals tested being positive for Bd. For these individuals, Bd infection intensity was relatively low, consistent with non-epizootic conditions [51, 67], indicating host resistance mechanisms rather than tolerance of high infection loads. Our study also shows that skin microbial communities and skin defense peptides inhibited the growth of the pathogen and thus, could explain host–pathogen coexistence in these two species of high Andean frogs and may also explain a mechanism for surviving species in other parts of the Andes where epizootics have eliminated many Bd-susceptible species [68, 69]. However, since bacteria and peptides samples were collected in different surveys and from different individuals, we could not test the hypothesis that these two components

are acting together. In this study, life stage, rather than frog species, was the best predictor of the bacterial community composition. We suggest that integrative studies of multiple defense mechanisms, their interactions, and their variation across life stages (e.g., [70]), such as we present here, are necessary to understand why some species may survive epizootics. By integrating various approaches to untangling complex host–pathogen–microbiota dynamics within a single study system, our research highlights the importance of using a more holistic approach to better understand disease outcomes in diverse species and environments and inform possible mitigation efforts.

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Compliance with ethical standards

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

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