Molecular Ecology

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MOLECULAR ECOLOGY

ORIGINAL ARTICLE OPEN ACCESS

Skin Microbiome Variation Among Hosts and *Batrachochytrium dendrobatidis* Infection in *Hyla meridionalis* and *Pelophylax perezi*

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Received: 26 December 2025 | **Revised:** 19 March 2026 | **Accepted:** 12 May 2026

Keywords: Bd-inhibitory bacteria | chytridiomycosis | *Hyla meridionalis* | nanopore sequencing | *Pelophylax perezi* | skin microbiome

ABSTRACT

Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd), is a key driver of global amphibian declines. The amphibian skin microbiome, which may include Bd-inhibitory bacteria, plays a crucial role in defence against infection, influencing host susceptibility and disease outcome. In this study, we investigated the skin microbiota and Bd infection in two frog species, *Hyla meridionalis* (Hm) and *Pelophylax perezi* (Pp), from northeastern Spain using full-length 16S rRNA gene sequencing. We found that microbiota composition differed significantly between frog species, with Pp harbouring greater bacterial richness and a community composition more similar to the aquatic environment than Hm. Asymptomatic Bd infection did not significantly alter microbiota diversity or community composition in either species. Nonetheless, differential abundance analyses revealed distinct bacterial taxa associated with host species and, to a lesser extent, with Bd infection. The composition of putative Bd-inhibitory bacteria also differed between frog species but was not influenced by Bd, suggesting that the microbiome's protective role may not be straightforward. Lower pathogen loads in Pp suggest a potential link between microbial richness and disease resistance. Our findings indicate that amphibian skin microbiota do not necessarily shift in response to Bd infection in asymptomatic hosts but may play a role in species-specific mechanisms of tolerance and resistance. Additionally, we detected potentially pathogenic bacteria of public and animal health concern on amphibian skin, highlighting amphibians as potential reservoirs and sentinels of ecosystem and public health. Overall, our findings indicate that amphibian skin microbiomes are shaped primarily by host species identity rather than asymptomatic Bd infection, emphasizing the importance of baseline microbiome variation in understanding host-pathogen dynamics and informing probiotic bioaugmentation strategies.

1 | Introduction

Amphibians are the most threatened vertebrates on Earth, with over 41% of species at risk of extinction (IUCN 2024). A major driver of global amphibian declines is chytridiomycosis, a disease caused by the chytrid fungi *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal) (Longcore et al. 1999; Martel et al. 2014). These multi-host pathogens have been linked to the decline of over 500 amphibian species and the extinction of 90, representing the greatest loss of biodiversity due to disease ever recorded (Scheele et al. 2019). Nevertheless, the outcomes of exposure and infection vary significantly between host species, populations and life stages (Grogan et al. 2023). These differences are explained by environmental, pathogen

and host factors. Among host factors, the immune response is key in determining variations in resistance and tolerance to pathogens.

The pathogenicity of amphibian chytrid fungi is based on their capacity to infect and disrupt the vital functions of amphibian skin. Consequently, the skin and its microbiome are the first line of defence and such constitutive immune components are crucial determinants of host susceptibility and disease outcomes (Bernardo-Cravo et al. 2020; Grogan et al. 2023). The amphibian skin microbiome can contain bacteria that repel, prevent colonization and/or inhibit Bd and Bsal growth by producing antifungal metabolites (Grogan et al. 2018; Woodhams et al. 2015). Microbiota can also directly compete with Bd and Bsal for resources, enhance host immune responses and support physiological functions (Grogan et al. 2018). Understanding the composition and variation of skin microbiomes among species and populations is, therefore, crucial for anticipating chytridiomycosis susceptibility and outbreak emergence. Additionally, identifying protective microbial communities may allow manipulation of the skin microbiome in susceptible amphibians (Garner et al. 2016). Probiotic bioaugmentation has been advocated as one of the few sustainable strategies for chytridiomycosis prevention and treatment, promoting amphibian conservation (Bletz et al. 2013; Harris, Brucker, et al. 2009; Harris, Lauer, et al. 2009; Kueneman et al. 2016).

Differences in the diversity and composition of skin microbial communities can be explained by both amphibian intrinsic and extrinsic characteristics (reviewed by Bernardo-Cravo et al. 2020; Jiménez and Sommer 2017). Intrinsic characteristics include host factors such as species identity, life stage, sex, genetics, immune status, behaviour or diet. Extrinsic characteristics represent biotic and abiotic environmental traits, including sympatric species and community composition, habitat, or climatic conditions that serve as a source pool of bacterial exposure. In addition, habitat loss, exposure to pollutants, or other anthropogenic disturbances are also known to alter skin microbiome, besides having other health and fitness consequences (Jiménez and Sommer 2017). More generally, factors affecting the overall health status of the host, such as the presence of potential pathogens, can lead to shifts in skin microbiota.

As parasites with a tropism for the skin of post-metamorphic stages, Bd and Bsal can conceivably disturb the diversity and composition of amphibian cutaneous microbiota. Previous studies found that Bd infection altered the skin microbiome of frogs under both experimental and field conditions (Jani and Briggs 2014; Walke et al. 2015). By sampling pre-epizootic populations, Jani et al. (2017) showed that bacterial community composition in frog skin was more closely associated with Bd infection loads than with disease outcomes. The authors, therefore, suggested that differences in microbiome composition were not conferring prior resistance and were a consequence rather than a cause of Bd establishment and load. Altogether, there is good evidence that Bd infection triggers detectable changes in skin microbiome composition and function in diverse amphibian species. However, distinguishing between microbiome-induced resistance and Bd-induced disturbance when evaluating differences in microbiome is not straightforward. Inferring cause or effect requires longitudinal studies replicated across multiple scenarios, and both responses are not mutually exclusive (Jani and Briggs 2014).

Regardless of whether microbiome alterations precede or result from infection, studying the relationship between Bd and skin microbiomes in multiple amphibian species is essential to improve chytridiomycosis monitoring and management. Moreover, establishing baseline data and tracking wildlife microbiomes can have broader applications for human, domestic animal and ecosystem health (Ribas et al. 2023). Monitoring amphibian-associated microbiomes might help to foresee zoonotic disease outbreaks as amphibians can harbour pathogens significant to public health (Mettee Zarecki et al. 2013; Mitchell 2011), and they thrive in human-modified environments. Amphibians are highly sensitive to environmental change, and monitoring their microbiomes has been proposed as an early indicator of ecosystem degradation and overall ecosystem health (Ribas et al. 2023).

In this study, we analysed the skin microbiota of two European frog species, *Hyla meridionalis* (Hm) and *Pelophylax perezi* (Pp), inhabiting an urban environment in northeastern Spain using high-throughput amplicon sequencing of the full-length 16S rRNA gene. Our goals were to (1) describe and compare the diversity and composition of skin bacterial assemblages among frog species and environmental (water) microbiota, (2) evaluate the prevalence of putative Bd-inhibitory bacteria in both frog species, (3) analyse whether Bd infection is associated with variations in skin microbiota diversity and composition and (4) assess the presence of potentially pathogenic bacteria of human and animal health concern on amphibian skin. To our knowledge, this is the first study reporting Bd infection, describing skin microbiota and analysing their associations in these species.

2 | Materials and Methods

2.1 | Study Site

The amphibian species under study were based at Montjuïc Park (41°36′71″ N, 2°16′47″ E) in Barcelona city, Northeastern Spain. This urban habitat comprises a main pond connected to a series of 30 smaller ponds. All ponds are human-made and contain abundant aquatic vegetation, dominated by water lilies. Around the ponds, the park includes paved footpaths, grass pitches and numerous tree, bush and plant species. The park sustains populations of three anuran species: *Hyla meridionalis* (Hm, Family Hylidae), *Pelophylax perezi* (Pp, Family Ranidae) and *Alytes obstetricans* (Family Alytidae). Bd infection has been previously detected through qPCR (Blooi et al. 2013) in post-metamorphic individuals of the first two species without clinical signs of disease (Ribas M.P., unpublished results).

2.2 | Field Sampling

Animal capture, manipulation and sampling protocols were approved by Departament d'Acció Climàtica, Alimentació i Agenda Rural (Generalitat de Catalunya, Spain) under the licence SF/0144/24.

Field sampling took place on the 20th of May 2024, coinciding with the reproductive season of the study species and to limit the impact of seasonality on microbiome and infection data. We focused our study on adult individuals of two frog species: Hm and Pp. Frogs were located through calls and visual encounters. *Hyla meridionalis* has mainly an arboreal lifestyle and was captured by hand from the nearby vegetation, whereas Pp are generally aquatic species and were captured using dipnets. Each animal was contained in a new plastic bag and handled with a fresh pair of nitrile gloves to prevent pathogen transmission and sample contamination. We swabbed the skin of each frog in duplicate using sterile dry swabs (Snappable Ps + cotton, Deltalab) according to a standardized protocol that targets the ventral region and limbs (Hyatt et al. 2007). Frogs were thoroughly examined for signs of disease before being released back at their capture site. Skin swabs were placed in sterile 1.5 mL cryovials and stored within the first 6 h after collection at −20°C until DNA extraction.

To characterize the microbial community composition of the aquatic environment, we collected 200 mL of pond water in triplicate. Each sample was filtered through a 0.45 μm Whatman filter and DNA was extracted within the first 24 h.

2.3 | Amphibian Chytrid Fungi Diagnostics

We extracted DNA from the first skin swab following the method described by Hyatt et al. (2007). Briefly, 65 μL of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, USA) and 30–40 mg of silica beads (0.5 mm, Scientific Industries Inc.) were added to each sample. The sample was homogenized for 2 min in a TissueLyser II (QIAGEN), incubated for 10 min at 99°C, and centrifuged at 13,000× g for 3 min. The supernatant was then recovered and stored at −80°C until analysis. Extractions were diluted 1:5 with nuclease-free water before qPCR assay to reduce the effects of potential inhibitors (Boyle et al. 2004; Hyatt et al. 2007). To determine chytrid infection status and quantify pathogen loads, we performed a duplex qPCR to simultaneously detect Bd and Bsal on each animal sample (Blooi et al. 2013). A synthetic double-stranded 750 bp gBlocks (Integrated DNA Technologies, USA) gene fragment containing the target PCR sequences of both pathogens was used as a positive control and quantitation standard. Standard dilutions in log10 increments (10^{-1} to 10^{6} copies; in duplicate) were included in each qPCR plate to create a standard curve, allowing us to quantify the gene copy number per reaction. Negative controls (nuclease-free water) were also included in each plate. To determine final pathogen loads per PCR reaction, obtained copy numbers were multiplied by the dilution factor (i.e., 5). A sample was considered positive if the gene copy number per reaction was above 1. All qPCR assays were run on an Applied Biosystems 7500 Real-Time PCR System.

2.4 | 16S rRNA Amplicon Sequencing and Library Preparation

We extracted DNA from duplicate skin swabs and filters using the QIAmp PowerFecal Pro DNA Kit (QIAGEN, Germany), following the manufacturer's instructions. The duplicate swab of 12 Bd-positive and 12 Bd-negative Hm, and 7 Bd-positive and 7 Bd-negative Pp was used for skin microbiota analysis. All Bd-positive individuals were included in the microbiota study, and negative samples were randomly selected from all qPCR negative swabs.

Bacterial 16S rRNA was amplified by PCR using the SQK-16S114.24 Barcoding Kit (Oxford Nanopore Technologies, ONT, UK) containing the 27F/1492R primer set, which enabled the amplification of the entire ~1500 bp 16S rRNA gene. Each 50 μL reaction contained 25 μL of LongAmp Hot Start Taq 2× Master Mix (New England Biolabs, USA), 10 ng of genomic DNA sample and 10 μL of barcoded primers at 1 μM each. The temperature and cycling conditions were as follows: a denaturation step of 95°C for 1 min; followed by 25 cycles at 95°C for 20 s, 55°C for 30 s and 65°C for 2 min; and a final extension at 65°C for 5 min. Sample DNA was quantified using a Qubit 4 Fluorometer and the dsDNA High Sensitivity Assay (Invitrogen, USA) before and after PCR amplification.

Library was prepared following the manufacturer's protocol for the ONT SQK-16S114.24 Barcoding Kit. Briefly, 10 ng/μL of the full-length 16S product of each sample was used as starting material and the final library with 50 fmol of DNA was loaded on a new R10.4.1 flow cell (FLO-MIN114, ONT). Real-time base-calling was performed on MinION Mk1C with the MinKNOW software (v.24.02.16) (both ONT), with Q < 8 (default threshold), and base-calling with Dorado (v. 7.3.11) (ONT).

2.5 | Data Processing and Statistical Analyses

Nanopore raw reads were analysed with Spaghetti pipeline (https://github.com/adlape95/Spaghetti). Adapters and barcodes were trimmed with Porechop (v 0.2.4), reads were filtered by length (1.200–1.800 bp) with Nanofilt (v.2.8.0) and quality check with Nanostat (v 1.6.0). Chimera removal was carried out with ycard (v 1.0.0) (Marijon et al. 2020) and taxonomic assignment with minimap2 (v. 2.26) (Li 2021), using Silva SSU 138 database (Chuvochina et al. 2025). Filtered reads were assigned through scripts included in the pipeline (Latorre-Pérez et al. 2021) and relative abundance tables were then obtained. In addition, Nanopore data was aggregated at the genus and species taxonomic level for the different analyses, using the function tax_glom from phyloseq package in R. This aggregation sums the abundances of all reads assigned to the same genus or species, reducing taxonomic redundancy and providing a more robust biological unit for analysis.

Alpha diversity was estimated by the number of single reads by phyloseq package (v 1.46) (McMurdie and Holmes 2013) and were studied at the genus level between sample types. Alpha diversity was assessed using the observed feature (richness) and Shannon (evenness) indices. These indices were analysed with linear regression models implemented in the lmtest package (v 0.9–40). Each model included one of the alpha diversity indices as the response variable and frog species, Bd infection status and Bd load as predictors. Statistical significance was evaluated using *F*-test. To visualize how variables (sample type, Bd

infection status and Bd load) influenced microbial community structure, we performed redundancy analysis (RDA) on centered log-ratio (CLR) transformed genus-level abundances using the *rda* function in the *vegan* package (v 2.7–1). The significance of the db-RDA axes and individual explanatory variables was assessed using permutation tests (anova.cca, 999 permutations). For explanatory variables showing significant effects and containing multiple levels (i.e., sample type), we conducted pairwise post hoc comparisons using PERMANOVA with Benjamini-Hochberg correction for multiple testing. Abundance profiles and taxonomic signatures were carried out with MicrobiomeStat package (v 1.2.0) (Lu et al. 2023).

Differential analysis was performed to evaluate differential genera among amphibian species and Bd infection status using Analysis of Compositions of Microbiomes with Bias Correction (ANCOMBC) using ANCOMBC package (v 1.4.0). Differential abundance was assessed on taxa with a prevalence over 10% at the genus level. Taxa were considered differentially abundant if they satisfied both q-value <0.05 (False discovery rate correction) and diff = TRUE. Custom figures were created using ggplot2 (v. 3.5.1), ggrepel (v. 0.9.5), gridExtra (v. 2.3), plotly (v. 4.10.4) and ggsignif (v. 0.6.4). Core microbiome (taxa shared by 100% of samples) at the genus level was identified using a Venn diagram obtained through https://www.interactivenn.net. Water was not included in the alpha diversity, differentially abundant taxa and core microbiome analysis due to the low sample replication.

Prevalence of Bd infection was estimated from the ratio of positive to the total number of samples with Wilson confidence intervals of 95%. Differences in prevalence among species were assessed using a Pearson Chi-squared test. Log-transformed Bd loads were compared among species using Student's *t*-tests. All data visualization and statistical analyses were performed using R version 4.1.4 (R Core Team 2024) and significance thresholds set at $p \leq 0.05$.

2.6 | Putative Bd-Inhibitory Bacteria Analysis

Taxonomically assigned sequences from the Spaghetti pipeline were extracted for mapping against the database of putative Bd-inhibitory bacteria (Woodhams et al. 2015), using minimap2 with default parameters. The resulting files were filtered with a strict threshold to include only high-quality alignments, keeping only mapped sequences with a minimum alignment length of 500 bp, a minimum alignment of 80% of the total target sequences, and a mapping quality score of 60 (highest mapQ).

The relative abundance and the proportion of taxa classified as 'putative Bd-inhibitory' were calculated for each animal and environmental (water) sample. Here, proportion refers to the ratio of inhibitory taxa to the total number of taxa, whereas relative abundance represents the fraction of sequencing reads assigned to inhibitory taxa. We report both metrics because they capture complementary ecological patterns. For each sample, we then calculated a log-ratio metric representing the relative representation of putative Bd-inhibitory bacteria, defined as the logarithm of the ratio between the total read abundance of Bd-inhibitory taxa and the total abundance of all remaining taxa. This transformation accounts for the compositional structure of microbiome data. Differences in this log-ratio metric among sample types were tested using Kruskal-Wallis tests, followed by Dunn's post hoc tests. Differences between infected and uninfected individuals were assessed using Wilcoxon rank-sum tests.

2.7 | Potentially Pathogenic Bacteria Analysis

Bacteria of public and animal health concern were selected from our reads database based on the bacterial diseases listed by the European Centre for Disease Prevention and Control (ECDC 2024), the bacterial priority pathogens list and bacteria of potential health concern by the World Health Organization (WHO) (Lightfoot 2003; WHO 2024) and the World Organization for Animal Health list of notifiable diseases (WOAH 2024a; 2024b) (Table S2). Barplots of the selected bacteria's relative abundance were built for Hm, Pp and water samples with R software using the above packages.

3 | Results

We collected a total of 95 skin swabs in duplicate from the two frog species (Hm = 46 and Pp = 49). We found evidence of Bd infection via qPCR in 19 individuals, resulting in an overall infection prevalence of 20% (95% CI: 13.19–29.14), and species prevalence of 26.09% (12/46, 95% CI: 15.60–40.26) and 14.29% (7/49, 95% CI: 7.10–26.67) for Hm and Pp, respectively. Differences in Bd prevalence among amphibian species were not statistically significant. The median pathogen load among infected individuals was 39.25 copies (range 4.18–10,700.62 copies), and pathogen loads were significantly higher in Hm than in Pp ($p = 0.0082$, Figure S1). All animals were Bsal-negative and evaluated as clinically healthy.

The entire 16S rRNA gene of the skin microbiota was sequenced for 38 individuals, 24 Hm (12 Bd-positive, 12 Bd-negative) and 14 Pp (7 Bd-positive and 7 Bd-negative), and 3 water samples (Table S1). Long-read sequencing samples using the Nanopore MinION platform produced an average of 7.58 Gb of estimated bases and 5,120,000 raw reads. After filtering and quality control, sequence length had an average of 1485.40 bp and quality control of 10.23. Once the reads were aligned against the Silva reference database, a total of 3182 taxa collapsed at the genus level. Furthermore, by filtering with a frequency greater than three and 20% prevalence among samples, a total of 500 taxa were obtained at the genus level. The percentage of taxonomic assignment at the genus level was an average of 99.3% between all three sample groups (Table S1).

3.1 | Taxonomic Composition

Proteobacteria was the most abundant phylum (> 87%) in all frog species, regardless of infection status, as well as in water samples (Figure 1A). Cyanobacteria, Firmicutes and Bacteroidota were also present at high abundances across all groups, albeit to a different extent depending on the type of sample (0.26%–3.47%). Interestingly, Campilobacterota was the second most abundant phylum in water (9.81%) but was only marginally found in Pp (0.73%) and Hm (0.02%) samples.

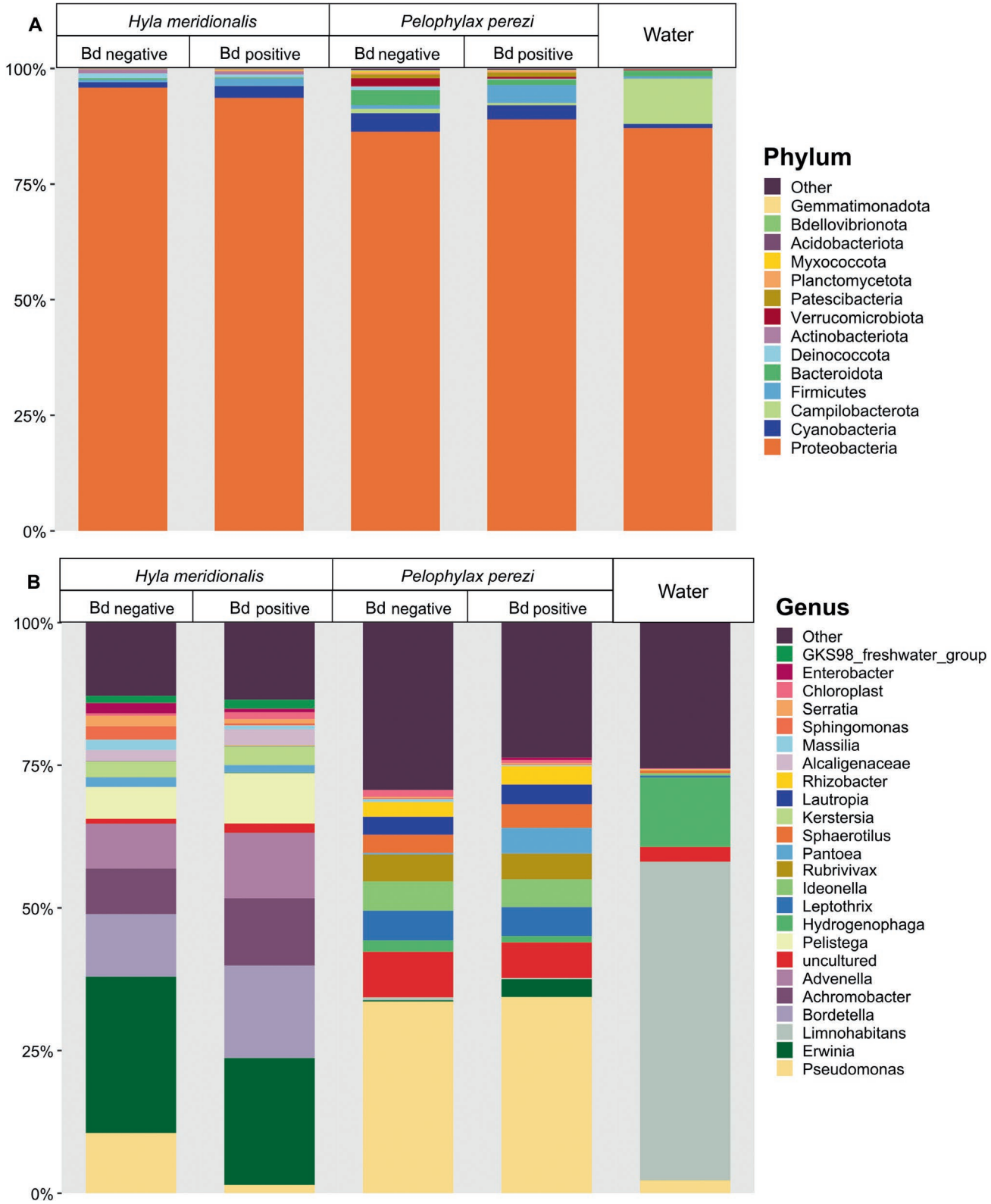

FIGURE 1 | Relative abundance of (A) the top 15 most abundant bacterial phyla and (B) top 25 most abundant bacterial genera across frog species (*Hyla meridionalis* and *Pelophylax perezi*), infection status (Bd negative and Bd positive), and in the water environment. Bd, *Batrachochytrium dendrobatidis*.

At the genus level (Figure 1B and Data S1), remarkable differences were observed among frog species. On one hand, *Erwinia* (24.92%), *Bordetella* (13.47%), *Achromobacter* (9.84%), *Advenella* (9.57%), *Pelistega* (7.15%) and *Pseudomonas* (6.17%) were the most abundant genera in Hm. On the other hand, *Pseudomonas* (31.91%) was the most abundant genus in Pp, with all other bacterial genera found at less than 5% of relative abundance. Water samples showed a divergent pattern, being dominated by

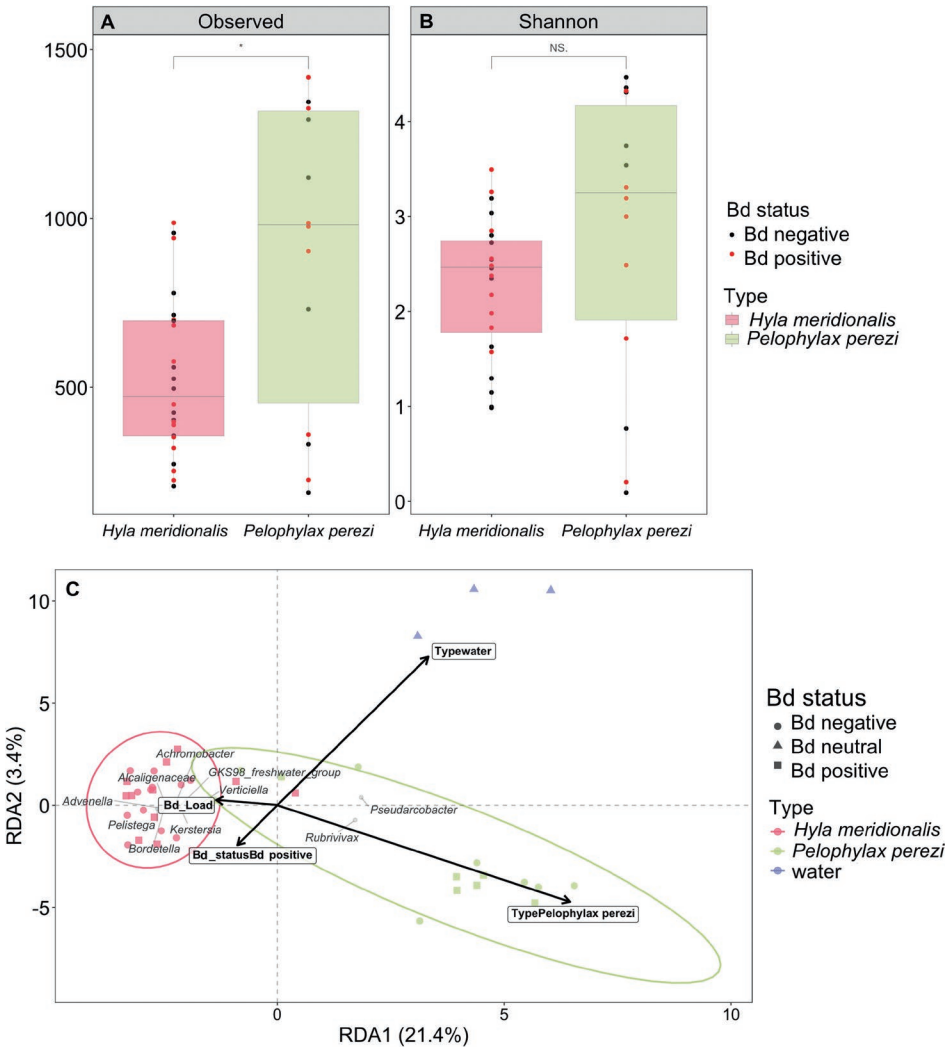

FIGURE 2 | Alpha diversity measures of *Hyla meridionalis*, *Pelophylax perezi* samples at the genus taxonomic level. (A) The observed feature index was used to measure bacterial richness and (B) the shannon index was used as a combined measure of evenness. Asterisks indicate statistically significant differences ($p \leq 0.05$); NS, non-significant differences. (C) Distance redundancy analysis (RDA) based on CLR transformation between type, Bd status and load. Samples are colour-coded according to type (*Hyla meridionalis* in red; *Pelophylax perezi* in green; water in blue) and shape according to Bd status (Bd negative in cercle; Bd neutral in pyramid; Bd positive in square). Black arrows indicate the direction and strength of the explanatory variables on the plot. Genera are labelled in grey next to their respective arrows and circles. Bd, *Batrachochytrium dendrobatidis*.

Limnohabitans (55.78%), followed by *Hydrogenophaga* (12.19%), *Pseudarcobacter* (7.67%), *Rhodoferax* (3.11%) and *Pseudomonas* (2.30%).

3.2 | Diversity Analyses

Significant differences in alpha diversity were only found in the observed feature index among Hm and Pp ($F = 9.873$; $p = 0.003$), where Pp samples presented higher richness (estimate = 366.3 ± 116.6 std). No significant effect was detected in Bd status or load for any alpha diversity metrics (Figure 2A,B and Data S2).

On the other hand, the RDA model for microbiota composition was significant ($F = 3.433$, $p = 0.001$, constrained proportion 27.61%). Specifically, the variable sample type (Hm, Pp and water) had a significant effect on community composition ($F = 6.165$, $p = 0.001$), while Bd infection status and load were not significant ($p > 0.05$). In addition, permutation tests by axis revealed that only the first canonical axis (RDA1) was significant ($F = 10.40$, $p = 0.001$), while the remaining axes were not ($p > 0.05$) (Figure 2C and Table S3). Pairwise PERMANOVA analyses revealed significant differences in community composition between Hm and Pp ($F = 9.09$, $p = 0.001$), as well as between Hm and water samples ($F = 6.09$, $p = 0.001$). In contrast, no significant differences were detected between Pp and water ($F = 1.40$, $p = 0.148$) (Table S4).

3.3 | Differential Abundance Analyses

Based on ANCOM-BC analysis, several bacterial genera were identified as being significantly different among frog species (Figure 3A and Data S2). Specifically, 355 bacterial genera were identified as the most significant contributors to explaining the observed differences in bacterial composition. Of these, 277 were differentially more abundant in Pp, while 78 were more

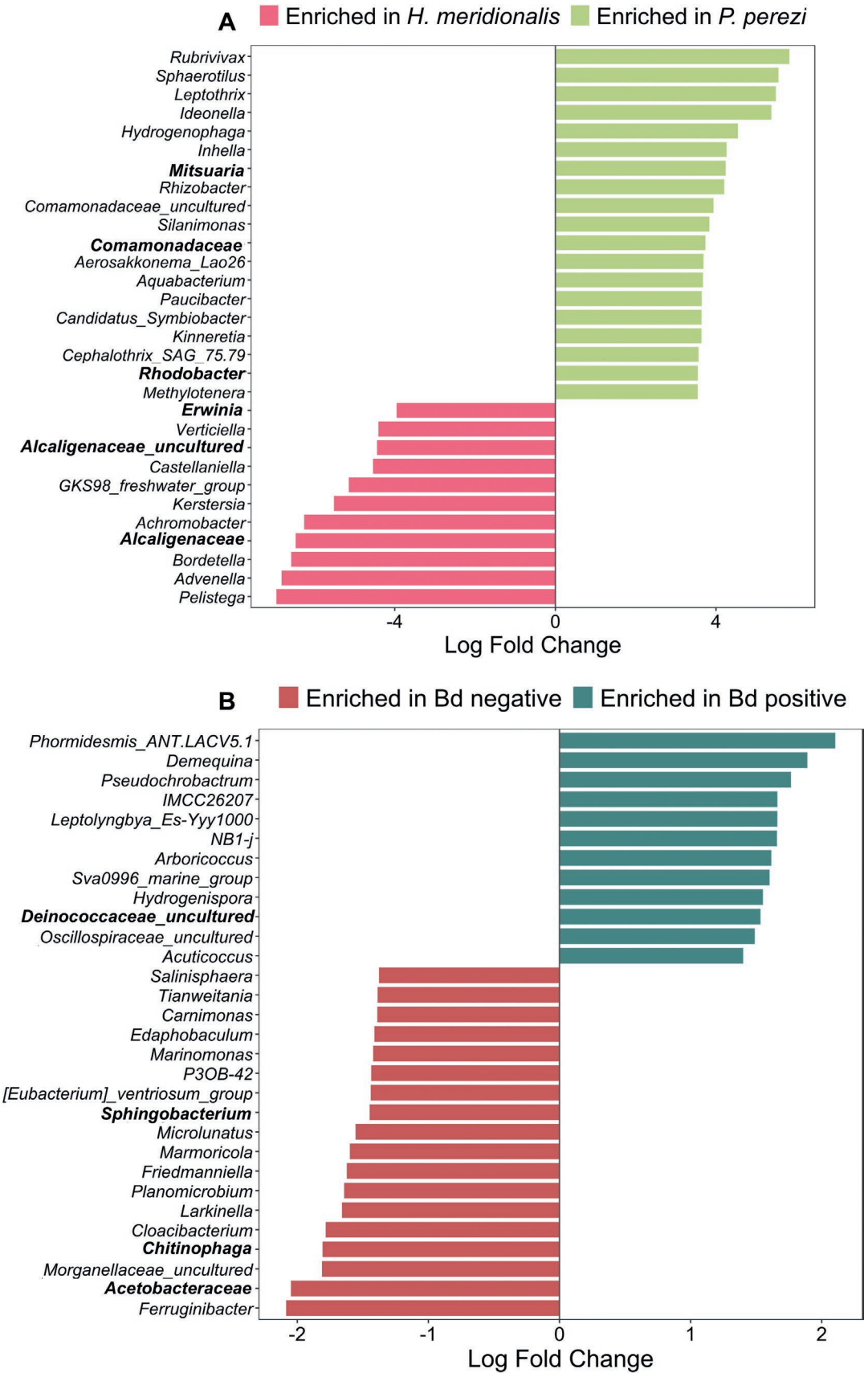

FIGURE 3 | Boxplots of the top 30 significantly differentially abundant bacterial genera between (A) *Hyla meridionalis* and *Pelophylax perezi*, and (B) Bd-negative and Bd-positive individuals across both frog species. Bolded genus names correspond to taxa identified as putative Bd-inhibitory bacteria. The *x*-axis shows the magnitude of differences between groups ($\log_{10}$ fold change), and the *y*-axis lists genera with significant differences ($p \leq 0.05$).

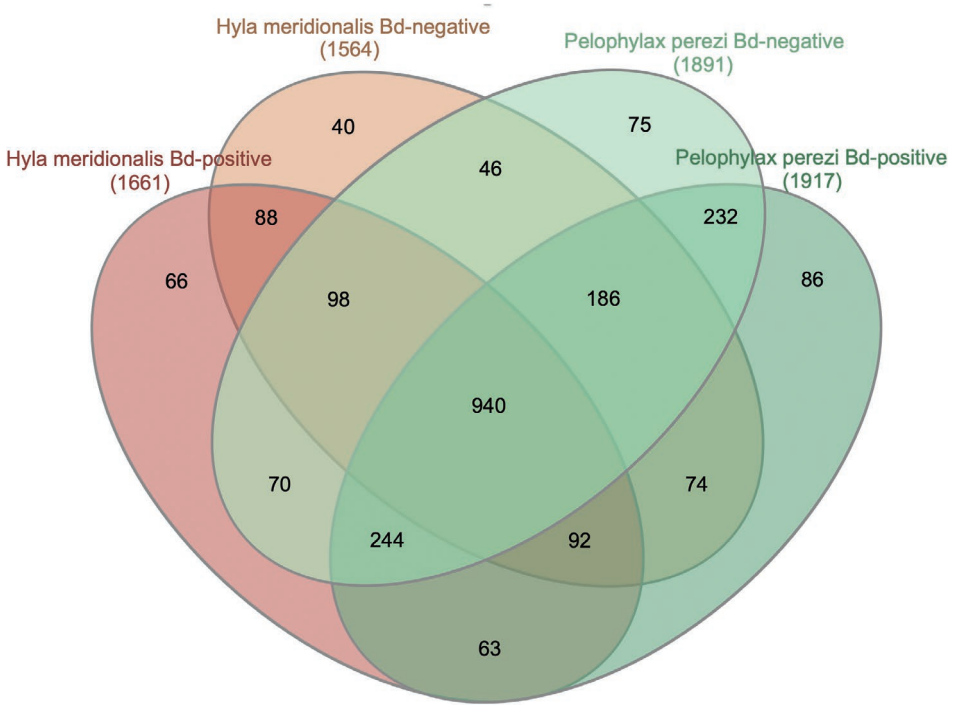

FIGURE 4 | Venn Diagram of bacterial genera shared across and exclusive to each sample type. Sample types include frog species (*Hyla meridionalis* and *Pelophylax perezi*) and *Batrachochytrium dendrobatidis* (Bd) infection status (Bd-positive and Bd-negative), resulting in four scenarios studied.

abundant in Hm. The top differentially abundant genera in Hm were *Alcaligenaceae, Pelistega, Advenella, Achromobacter, Bordetella and Kerstesia*. On the other hand, *Rubrivivax, Sphaerotilus, Leptothrix, Ideonella* and *Hydrogenophaga* were the most distinct in Pp.

In addition, we identified 85 bacterial genera associated with Bd status across both frog species, with 33 enriched in Bd-positive individuals and 52 enriched in Bd-negative individuals (Figure 3B and Data S3). Examples of taxa enriched in Bd-positive samples include *Phormidesmis, Demequina* and *Pseudochrobactrum*, whereas *Ferruginibacter, Chitinophaga* and *Cloacibacterium* were enriched in Bd-negative samples.

3.4 | Core Microbiome

Core community analysis showed that 940 genera were shared across all four sample types studied (Hm and Pp, Bd-positive and Bd-negative) (Figure 4). In addition, 88 genera were found exclusively in Hm and 232 in Pp. Across both frog species, Bd-positive individuals harboured 63 unique genera, whereas Bd-negative individuals harboured 46.

3.5 | Putative Bd-Inhibitory Bacteria

Most samples, including animal and environmental (water) samples, exhibited an approximate proportion of putative Bd-inhibitory taxa of 50%, but the relative abundance of these taxa varied across samples, from 0.70% to 99.78% (Figure 5 and Figure S2). Analysis of the log-ratio of putative Bd-inhibitory bacteria revealed significant differences among the three sample types (Kruskal-Wallis $\chi^2 = 7.78$, df = 2, $p = 0.020$). Post hoc Dunn's tests indicated that Hm harboured a significantly higher relative representation of inhibitory bacteria than Pp (adjusted $p = 0.017$). In contrast, differences between frogs and water samples were not significant (adjusted $p > 0.68$ for both comparisons). Additionally, within-species comparisons between Bd-positive and Bd-negative individuals revealed no significant differences in log-ratios (Wilcoxon rank-sum: Hm $p = 0.10$; Pp $p = 0.81$), indicating that infection status was not strongly associated with the relative abundance of inhibitory taxa in our study. Finally, several putative Bd-inhibitory taxa were also identified as differentially abundant, occurring across both frog species and regardless of Bd infection status (Figure 3A,B).

3.6 | Potentially Pathogenic Bacteria

We identified 26 potentially pathogenic bacterial species in our study samples, as well as unassigned species from the genera *Aeromonas, Bordetella, Citrobacter, Clostridium, Enterobacter* and *Serratia*. Potentially pathogenic bacteria represented 8.68% of Hm skin microbiota, whereas only 1.54% of Pp skin microbiota and 0.24% of water microbiota (Figure 6). The most abundant bacteria in Hm were *Bordetella* sp. (4.06%), *B. pertussis* (1.52%) and *Serratia fonticola* (1.13%), followed by *Enterobacter* sp. (0.44%), *Serratia* sp. (0.43%) and *B. bronchiseptica* (0.3%). Conversely, the most abundant bacteria in Pp were *Clostridium* sp. (0.62%) and *Aeromonas veronii* (0.31%), with all other species < 0.2% of relative abundance. For water, *Aeromonas veronii* was the most abundant species (0.11%), followed by other *Aeromonas* spp. (all < 0.04% of relative abundance).

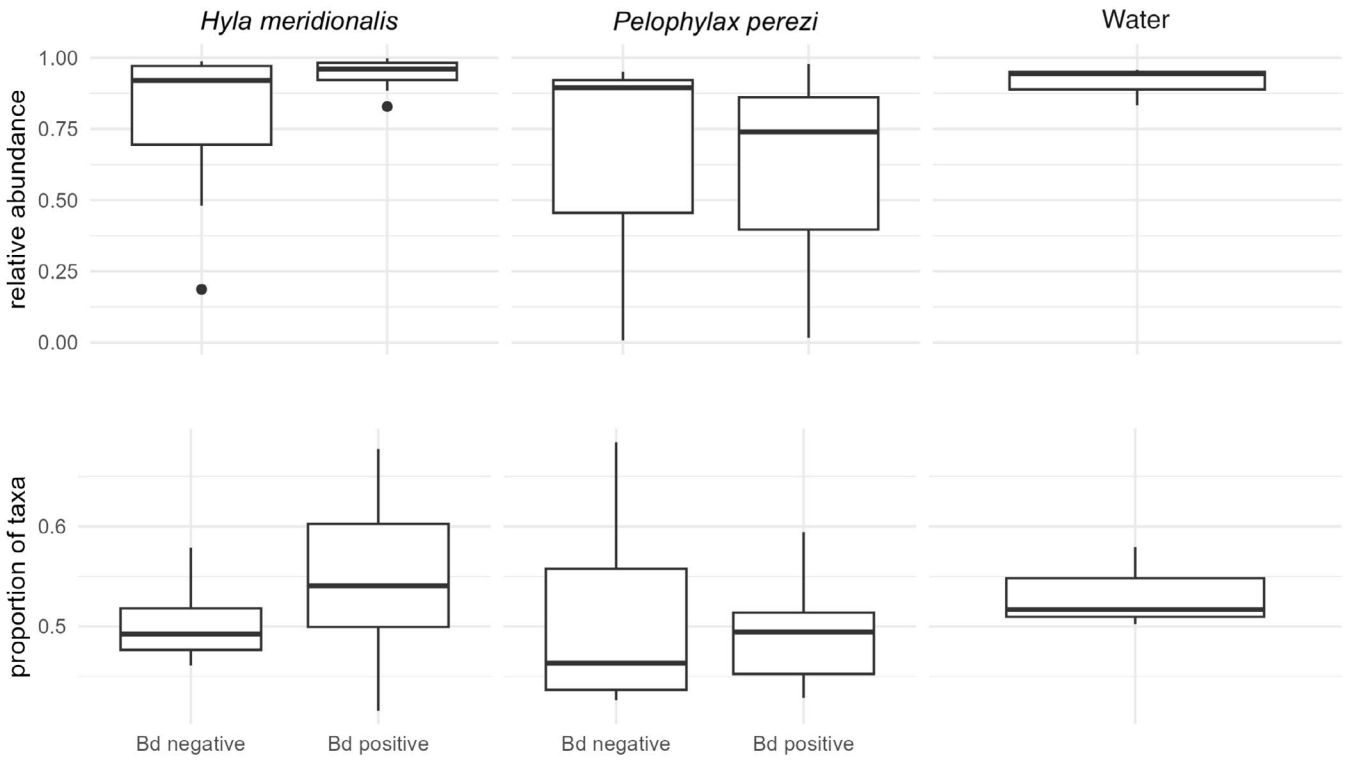

FIGURE 5 | Boxplots showing the fraction of sequencing reads belonging to putative *Batrachochytrium dendrobatidis* (Bd)-inhibitory bacteria taxa (relative abundance, top) and the ratio of taxa with putative Bd-inhibitory classification to the total number of taxa (proportion, bottom) in the skin microbiota of Bd-positive and Bd-negative *Hyla meridionalis* and *Pelophylax perezi* and in environmental (water) microbiota.

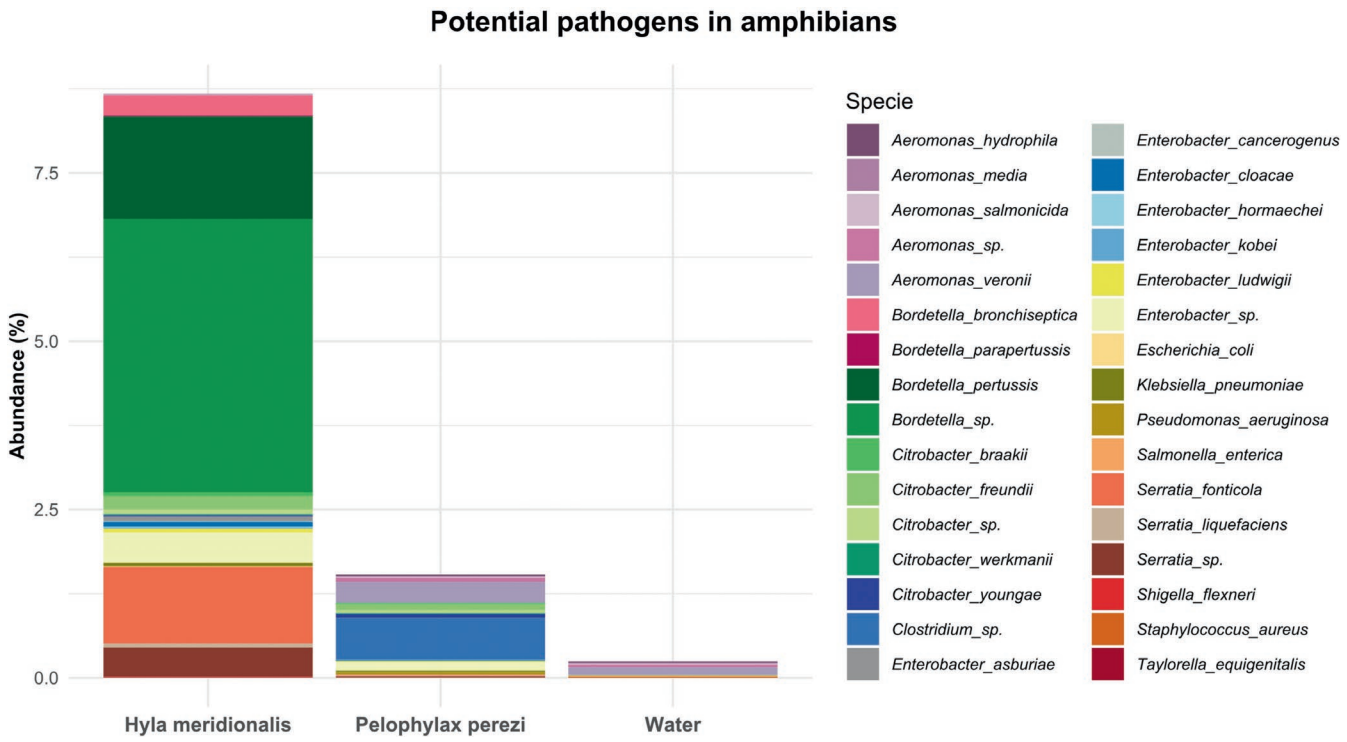

FIGURE 6 | Relative abundance (%) of the potentially pathogenic bacterial species identified in the skin microbiota of *Hyla meridionalis* and *Pelophylax perezi*, and in environmental (water) samples.

4 | Discussion

In this study, we evaluated the variation in skin microbiota among two European frog species and its relationships with asymptomatic Bd infection. Although high-throughput sequencing techniques are increasingly used to investigate amphibian skin microbiome, most studies focus on tropical species (Abarca et al. 2018; Bletz et al. 2017; Jiménez

et al. 2019; Mutnale et al. 2021; Rebollar et al. 2016; Schmeller et al. 2022), or temperate species from North America (Goodwin et al. 2022; Kruger 2020; Kueneman et al. 2014; Muletz Wolz et al. 2018; Ramírez-Barahona et al. 2023). Many of these studies are starting to focus their attention on non-susceptible hosts or populations in coexistence with Bd. Nevertheless, little is known about skin microbiome variations among European amphibian species and in response to Bd infection, with studies in postmetamorphic stages having been conducted only on a single highly susceptible species (Bates et al. 2022). For the first time, we report Bd infections without disease or mortality in Hm and Pp from northeastern Spain. By exploring the differential responses to asymptomatic Bd infection and variations in skin microbiota among these species, we contribute to a better understanding of the microbiome's protective role in chytridiomycosis.

4.1 | Skin Microbiota Varies With Host Species

We found distinctly different skin microbiotas between sympatric Hm and Pp. In agreement with other studies, we show that the skin bacterial composition of our study species was replete with Proteobacteria, followed by Firmicutes and Bacteroidota (Abarca et al. 2018; Bletz et al. 2017; Kueneman et al. 2014; Schmeller et al. 2022; Smith et al. 2023). Moreover, the relatively high abundance of *Pseudomonas*, particularly in Pp, is congruent with the findings reported for other frog species around the world (Bletz et al. 2017; Kueneman et al. 2014; Schmeller et al. 2022). While few bacterial taxa were exclusive to each species, we show significant differences in alpha diversity, community composition and differentially abundant taxa among frog species. This provides additional evidence that host species identity plays a crucial role in shaping the composition of the skin-associated bacterial microbiota (Abarca et al. 2018; Kueneman et al. 2014; McKenzie et al. 2012; Rebollar et al. 2016).

Nevertheless, microbiome variation among species may be more influenced by host ecology, behaviour and microhabitat selection than by host phylogeny (Bletz et al. 2017; Smith et al. 2023). Microhabitat preferences may expose amphibians to different environmental microbial pools, which affect the colonization and maintenance of the skin microbiota. Across studies, arboreal frog species appear to have the least bacterial diversity (alpha diversity metrics) compared to terrestrial or aquatic species (Bletz et al. 2017; Kueneman et al. 2014). Similarly, our aquatic species, Pp, harboured significantly greater bacterial richness than the arboreal Hm. The influence of the environmental pool appears evident in our RDA analysis, as no significant differences were detected between Pp and water samples. In contrast, the arboreal Hm showed significant differences from water, indicating a weaker influence of the aquatic environmental pool.

The differentially abundant genera in each frog species also suggest a substantial influence of microhabitat on skin microbiota. In Hm, the main significantly enriched genera belonged to the family Alcaligenaceae, which has consistently been associated with arboreal frog species (Bletz et al. 2017). Members of this family are found in water, soil and animal samples, but there is evidence that genera such as *Achromobacter* and *Advenella* have evolved in close association with plants (Kuzmina et al. 2022; Nascimento et al. 2021). This could explain their enriched abundance in the skin microbiota of arboreal frogs. Conversely, the differentially abundant genera *Sphaerotilus* and *Rubrivivax* in Pp belong to the family Comamonadaceae. This family was also abundant in the water samples of our study (genera *Limnohabitans* and *Hydrogenophaga*), reflecting the aquatic lifestyle and close association of Pp with stagnant water.

Both host and environmental factors probably drive the community composition of the skin microbiome of amphibians. Unfortunately, our study design did not allow us to investigate the influence of large-scale climatic and ecological variation. Our study species are broadly distributed across northeastern Spain, as well as other regions of the Iberian Peninsula and France. Replicating sampling in different geographic locations and habitats in the future would allow the examination of patterns in community composition across sites and elucidate the natural variation in skin microbiome within species. Additionally, environmental sampling should be extended to include various sample types (e.g., water, sediment, soil) and measurements to assess their relationship with host microbiomes. Regardless, our study lays the foundation for future research on skin microbiomes of European amphibian communities.

4.2 | Asymptomatic Bd Infection Is Not Associated With Major Shifts in Skin Microbiota

Neither Bd infection status nor infection loads were linked to major differences in the cutaneous bacterial composition of our study species. This was consistent across microbiota metrics, including alpha and beta diversity and core microbiome analyses. While differential abundance analysis was also unremarkable within Pp, significantly enriched bacterial taxa were identified between Bd-positive and Bd-negative Hm. However, these differential genera do not appear to follow a distinct pattern of characteristics that would explain their abundance in either infected or uninfected amphibians. Furthermore, Bd infection and load did not influence the composition of putative Bd-inhibitory bacteria in either frog species. Overall, skin microbiota showed little variation that could explain differential risk or response to Bd infection at an individual level. Other studies found a similar lack of association between Bd infection and bacterial composition in species with asymptomatic infections or populations with a long history of pathogen exposure (endemic infections) (Belden et al. 2015; Kruger 2020; Longo et al. 2015). Given that both host-associated microbiomes and Bd infections are dynamic throughout seasons and over time (Estrada et al. 2019; Longo and Zamudio 2017), longitudinal studies may be necessary to provide greater insight into these relationships. Importantly, all individuals in this population have likely been exposed to Bd, and therefore, our findings reflect patterns in an already exposed host population. Similar studies in naïve, non-exposed populations would be valuable to determine whether skin microbiota associations with Bd infection differ in populations without prior pathogen exposure.

On the other hand, variations in microbiome composition may explain distinct responses or defence mechanisms against Bd infection among host species. Animal defences against infection involve two complementary mechanisms: resistance and tolerance. Resistance refers to the host's ability to limit pathogen establishment or infection loads, whereas tolerance defines the ability to minimize the resulting pathology. Although Bd infections were asymptomatic in both of our study species, Pp harboured significantly lower pathogen loads than Hm. Moreover, despite aquatic species being generally more exposed to Bd infectious stages (Burrowes et al. 2017; Scheele et al. 2019), we observed similar prevalence in both species. Altogether, this suggests that Pp exhibits greater resistance to Bd, while the high pathogen loads coupled with the absence of pathology in Hm point towards a greater tolerance. Differences in resistance and tolerance among amphibian species can arise from variations in immune function, including both innate and acquired immune components (Grogan et al. 2018). Skin microbiome is part of the constitutive innate immunity against Bd and field studies have demonstrated consistently different skin microbial communities between Bd susceptible and resistant/tolerant species (Grogan et al. 2018; Rebollar et al. 2016). Notably, skin microbiome has been proposed as a major determinant of species resistance, as certain bacteria can prevent colonization and inhibit Bd growth (Grogan et al. 2023).

One characteristic of the skin microbiota that may account for the higher resistance of Pp to Bd infection observed in our study is bacterial richness. Richer microbial communities are generally considered more resistant and resilient to pathogen invasion (Lozupone et al. 2012; Rocca et al. 2019). This principle also seems consistent in Bd-amphibian systems, with studies demonstrating a correlation between higher microbial richness and reduced Bd growth or enhanced host-pathogen coexistence (Jani et al. 2017; Piovia-Scott et al. 2017). Higher microbial richness might confer resistance by increasing competition for resources and space with Bd and/or due to a greater probability that key protective bacteria are present. However, we would also have expected a greater proportion or abundance of Bd-inhibitory bacteria in Pp, whereas these bacteria appear more abundant in Hm. Although this pattern may be in response to Bd colonization as a mechanism to fight infection (Muletz-Wolz et al. 2019; Walke et al. 2017), the lack of association with Bd loads observed in our study suggests baseline differences in the skin microbiota among these frog species rather than supporting this hypothesis. More likely, our results highlight the limitations of using 16S rRNA sequence similarity to predict Bd-inhibitory function. Woodhams et al. (2015) database is based on antifungal properties detected on bacteria cultured in vitro but this may not reflect the in vivo expression of inhibitory functions. Further work will be required to determine how the putative Bd-inhibitory bacteria identified in the present study interact with Hm and Pp in their natural environments. Beyond bacterial community composition, other characteristics of the amphibian mucosome or skin-associated biofilms, such as mucus thickness or host-derived compounds, may also influence Bd colonization and infection dynamics (Chen et al. 2022; Jiménez et al. 2022). These factors were not evaluated in the present study and could contribute to interspecific differences in infection.

4.3 | Amphibian Skin Microbiota Contains Potentially Pathogenic Bacteria

By specifically searching for selected bacteria species, we show that amphibians can harbour important potential pathogens of human and animal health concern in their skin microbiota. Moreover, these bacteria appear in greater abundance in amphibian hosts than in environmental samples (water), highlighting their potential role as carriers. Of particular concern is our finding that Hm carries *B. pertussis* and *B. parapertussis* which can cause significant morbidity and mortality in children (Nieves and Heininger 2016). Nevertheless, most microbiome studies in amphibians do not report the presence of such pathogens as they are solely focused on amphibian health. Many amphibians thrive in urban and suburban environments (Hamer and McDonnell 2008; Konowalik et al. 2020), increasing the opportunities for exposure to human and domestic animal pathogens. Although amphibians may not be the primary hosts for such pathogens, they may act as reservoir and spillback hosts. Overlooking the close interconnection between wildlife, domestic animals and human health can hamper our ability to prevent and mitigate emerging diseases. Based on our findings, we propose that amphibians can serve as indicators of potentially pathogenic bacteria and anthropogenic disturbances in ecosystems. Therefore, monitoring amphibian-associated microbiomes can offer significant benefits beyond improving amphibian health. We strongly encourage future studies to specifically document the presence of potential pathogens to better understand and mitigate risks for wildlife, domestic animal and human populations.

4.4 | Conclusions

In this study, we describe for the first time the skin microbiota and its relationship with Bd infection in Hm and Pp, two widely distributed amphibian species in Western Europe. Understanding the microbial communities in different species and regions, as well as the intrinsic and extrinsic factors that drive variation is the first step towards developing strategies to mitigate chytridiomycosis. We show that skin microbiota diversity and composition vary between our two frog species but not with Bd infection in asymptomatic hosts. Moreover, we begin to elucidate the role of Hm and Pp in chytridiomycosis dynamics by comparing their relative resistance and tolerance to infection, linking these differences to variations in microbiota diversity and composition. Given the protective role of skin microbiome in chytridiomycosis, the study of resistant and tolerant host species may be an effective approach to identifying key bacterial taxa for probiotic candidate selection and bioaugmentation approaches in succumbing species. Additionally, we highlight the utility of tracking amphibian microbiomes as indicators of ecosystem health and emerging public health threats. Further research should expand on the factors driving variations in amphibian skin microbiome, test the Bd-inhibitory function of different bacterial taxa in amphibian hosts and natural environments through transcriptomic approaches, and develop microbial therapies for species threatened by disease.

Author Contributions

M.P.R.: conceptualization, methodology, validation, investigation, visualization, writing – original draft; C.T.: methodology, validation, formal analysis, data curation, visualization, writing – review and editing; M.V.J.: methodology, validation, formal analysis, writing – review and editing; L.M.-G.: resources, supervision, writing – review and editing; O.C.: conceptualization, investigation, resources, supervision, writing – review and editing. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

We thank Johan Espunyes, Xavier Fernández Aguilar, Laura Carrera, Mireia Blasco, Alessandra Kalantonis and Laura Canós for their support in sample collection. We are also grateful to Noemí E. Giler for her valuable assistance in the laboratory work. This manuscript is derived from research conducted as part of the doctoral thesis of Maria Puig Ribas (Universitat Autònoma de Barcelona, 2025), available through the institutional repository of the university.

Funding

This work was supported by Fundació Barcelona Zoo. Departament de Recerca i Universitats, Generalitat de Catalunya, Spain, FI_B 00171. CERCA Program.

Disclosure

Benefit-Sharing Statement: Benefits from this research accrue from the sharing of our data and results on public databases as described above.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information with the primary accession code PRJNA1123624. Access link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1138540?reviewer=1buf0losg5r4rkaoj8a5dl78ir. Full pipeline to perform the putative Bd-inhibitory bacteria analysis using 16S rRNA complete gene data sequenced through nanopore can be found at: https://github.com/MarietteViladomat/Amphibian-Skin-Microbiome-Bd-Inhibitory-taxa. We have shared the rest of our data as Supporting Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Samples used for microbiome analysis. For *Hyla meridionalis* and *Pelophylax perezi*, skin swabs were collected and the presence of chytrid fungi DNA and skin microbiome were assessed. Infection loads are expressed as gene copy numbers per reaction. Environmental (water) microbiome was analysed from filtered water samples, which were not tested for the presence of chytrid fungi. Accession numbers correspond to the sequence data deposited in the National Center for Biotechnology Information with the primary accession code PRJNA1123624 (Access link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1138540?reviewer=1buf0losg5r4rkaoj8a5dl78ir). **Table S2:** Results of linear models evaluating whether skin microbiota alpha diversity varied with sample type (*Hyla meridionalis* and *Pelophylax perezi*), *Batrachochytrium dendrobatidis* (Bd) infection status (positive or negative) and Bd load (numeric values). Asterisks indicate significant *p*-values ($p \leq 0.05$). **Table S3:** Results of redundancy analysis (RDA) assessing the influence of sample type (*Hyla meridionalis*, *Pelophylax perezi* and water), *Batrachochytrium dendrobatidis* (Bd) infection status (positive or negative) and Bd load (numeric values) on skin microbiota composition. Asterisks indicate significant *p*-values ($p \leq 0.05$). **Table S4:** Results of pairwise post hoc comparisons evaluating differences between multiple levels of the sample type variable used in the RDA. Hm = *Hyla meridionalis*; Pp = *Pelophylax perezi*d. **Table S5:** List of potentially pathogenic bacteria used to search in our database. Bacteria are classified by their relevance as recognized (R) or opportunistic (O) and by their primary host as human (H) or animal (A). This list has been created based on the bacterial diseases listed by the European Centre for Disease Prevention and Control (ECDC 2024), the bacterial priority pathogens list and bacteria of potential health concern by the World Health Organization (WHO) (Lightfoot 2003; WHO 2024) and the World Organization for Animal Health list of notifiable diseases (WOAH 2024a, 2024b). **Figure S1:** Boxplots showing $\log_{10}$-transformed *Batrachochytrium dendrobatidis* (Bd) loads for infected *Hyla meridionalis* and *Pelophylax perezi*. Significant differences in Bd loads among amphibian species are observed ($t = 2.9976$, $df = 16.837$, $p = 0.0082$). **Figure S2:** Relative abundance (top) and proportion (bottom) of putative *Batrachochytrium dendrobatidis* (Bd)-inhibitory bacteria in study samples grouped by amphibian species, Bd status and water samples. Bd+ = *B. dendrobatidis* positive; Bd− = *B. dendrobatidis* negative. **Data S1:** mec70393-sup-0002-DatabaseS1-S3.xlsx. **Data S2:** mec70393-sup-0002-DatabaseS1-S3.xlsx. **Data S3:** mec70393-sup-0002-DatabaseS1-S3.xlsx.