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BRIEF REPORT

ENVIRONMENTAL MICROBIOLOGY

The commensal skin microbiome of amphibian mountain populations and its association with the pathogen *Batrachochytrium dendrobatidis*

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Abstract

Microbial assemblages naturally living on the skin are an integral part of immunity. In amphibians, this skin microbiota may hold a mitigation solution against the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which causes the panzootic disease chytridiomycosis. We used 16S rRNA gene metabarcoding to test the adaptive microbiome hypothesis. We compared the community composition, richness, and putative Bd-inhibitory function of the skin microbiome of three amphibian host species in the Pyrenees, as well as three species in Taiwan, in both Bd-positive and negative mountain populations. In both geographical regions, the amphibian host species played a decisive role in shaping the microbial assemblage and putative anti-Bd properties. In the Pyrenees, the species most susceptible to chytridiomycosis, *Alytes obstetricans*, had the lowest relative abundances of putative protective bacteria. In Bd-positive and negative sites, individuals had different skin microbiomes, with all anuran species showing increased relative abundances of potential anti-Bd bacteria, while the Taiwanese caudata *Hynobius sonani* showed the opposite pattern. Our results suggest that, in response to exposure to the pathogen, the skin microbiota shifted to a defensive state with increased anti-Bd function, which may contribute to promoting disease resistance, as proposed by the adaptive microbiome hypothesis.

INTRODUCTION

The amphibian chytridiomycosis panzootic is responsible for mass amphibian die-offs worldwide and the decline of over 500 amphibian species, the greatest loss of biodiversity attributable to a disease ever recorded (Scheele et al., 2019). This emerging disease is caused by two waterborne fungal pathogens, the chytrids *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal). Since Bd and Bsal have been identified as major causes of amphibian decline and extinctions, microbial communities naturally residing on the amphibian skin have been a focus of intense research, in the hope that the amphibian skin microbiota holds a mitigation solution against chytridiomycosis (Bletz et al., 2013; Garner et al., 2016; Woodhams et al., 2011), as well as against other diseases such as ranaviruses (Harrison et al., 2019). However, our understanding of the associations between Bd and symbiotic skin communities remains limited, especially when it comes to microbial functional groups. Recent studies have examined correlations between the abundance of Bd-inhibitory taxa and Bd infection and shed new light on functional groups (e.g., Bates et al., 2022; Davis et al., 2017; Flechas et al., 2019; Goodwin et al., 2022; Jiménez et al., 2022; Nava-González et al., 2021).

In amphibians, the symbiotic skin microbiota is an integral part of immunity that benefits host health and provides a first line of host defence against pathogens, especially relevant for those of the skin like Bd (Bernardo-Cravo et al., 2020). First, it prevents exogenous microbes from adhering to the skin, settling and proliferating, through a competition for space and resources (Bernardo-Cravo et al., 2020; Martínez-Ugalde et al., 2024). Second, many amphibian skin bacteria and fungi actively secrete antimicrobial and antifungal metabolites that impede the growth of pathogens, as demonstrated for Bd in vitro (e.g., Bell et al., 2013; Harris et al., 2006; Niederle et al., 2019; Rollins-Smith, 2009; Ujszegi et al., 2023; Woodhams et al., 2015). In vivo, the symbiotic skin bacterium *Janthinobacterium* spp. that produces the metabolite violacein was transferred to the skin of the Bd-susceptible Mountain yellow frog (*Rana muscosa*). This bioaugmentation successfully decreased Bd infection and increased host survival, showing particular promise (Harris et al., 2009, but see Becker, Walke, Cikanek, et al., 2015; Becker, Walke, Murrill, et al., 2015). *Janthinobacterium* spp. was isolated from the redback salamander *Plethodon cinereus*. *P. cinereus* was targeted because it appears to be resistant to chytridiomycosis in nature (Becker & Harris, 2010). This is consistent with the idea that not all skin microbial communities exhibit the same ability to protect their amphibian hosts against pathogens. In general, the severity of infection depends, at least in part, on the composition and/or richness of the skin microbiome (e.g., Bates et al., 2018; Brucker et al., 2008; Jiménez & Sommer, 2017; Kueneman et al., 2014; Longo et al., 2015; Schmeller et al., 2022, but see Kruger, 2020). Species and populations resistant to Bd also have more protective skin microbial communities compared to hosts experiencing declines (Lam et al., 2010; Rebollar et al., 2016; Rollins-Smith, 2009; Woodhams et al., 2006, 2007).

Recently, Woodhams and collaborators have proposed the adaptive microbiome hypothesis to explain processes of disease resilience (Woodhams et al., 2023). According to this hypothesis, competitive microbial interactions couple with host immune responses to select for functions beneficial to the host (Woodhams et al., 2023). In the case of adaptive microbiome processes, exposure to a pathogen triggers the rapid proliferation of protective microbes and enriches the microbial community with anti-pathogenic members (microbial rescue) but transitorily decreases richness, explaining populational differences in skin microbiome communities and disease outcomes in the long-term (Woodhams et al., 2023). However, exposure to a pathogen can also cause a shift or disruption of the microbial community composition leading to altered host health, a process termed dysbiosis. In that case, the microbiota is unable to mediate the stress of the infection (because it is depauperated or because of homeostasis overload) which leads to a negative outcome (Woodhams et al., 2023). Several infection experiments, corroborated by field studies, have demonstrated that colonization of the skin by Bd and Bsal can lead to the establishment of an alternate stable skin microbiome (Bates et al., 2019; Jani et al., 2017; Jani et al., 2021; Jani & Briggs, 2014; Jani & Briggs, 2018). The latter may be caused by impairment of the skin osmoregulation and electrolyte transport due to Bd colonization (Longo et al., 2015), by a severe inflammatory response to Bd leading to the proliferation of opportunistic bacteria (Bates et al., 2018), or because the hosts developed an adaptive microbiome maintained as long as the pathogen is present (Woodhams et al., 2023).

In our study, we tested the adaptive microbiome hypothesis in two different mountainous regions, the Pyrenees and the Zhongyang Mountains in Taiwan. First, we took advantage of a long-term study system in the Pyrenees in which 22 high-altitude mountain lakes and ponds have been monitored for over 10 years for Bd presence, prevalence and infection intensity (Bates et al., 2018; Clare et al., 2016; Walker et al., 2010). We used 16S rRNA gene-metabarcoding to compare the community composition, dispersion, diversity, and putative Bd-inhibitory function of the skin microbiome of three anuran species with variable susceptibility to Bd infection and life-history traits, sampled over 3 years (2016–2018). The Common midwife toad (*Alytes obstetricans*) is highly susceptible to Bd infection and has suffered catastrophic declines in the Pyrenees, including mass-mortality events, that have led to local extirpations. In mountain populations, *A. obstetricans* can persist several years in the tadpole stage while remaining infectious. Those overwintering tadpoles are believed to be a reservoir of Bd infection, and thus are used as sentinels for Bd surveillance (Bates et al., 2018, Clare et al., 2016, Walker et al., 2010). *A. obstetricans* shares the habitat with the Spiny common toad (*Bufo spinosus*) and the Common frog (*Rana temporaria*). *R. temporaria* is usually the first to reach the lake for reproduction and is usually resistant to Bd infection, while *B. spinosus* has intermediate susceptibility to Bd (Clare et al., 2016).

In the Pyrenees, we focused on the tadpole skin microbiome because it is likely the stage in which infection occurs in the sentinel species *A. obstetricans*. As *A. obstetricans* larval skin is deficient in keratin and infection is restricted to the mouthparts, it is rarely acute or lethal. A previous study has monitored the trajectory of the skin microbiome of *A. obstetricans* tadpoles across host life history and among populations (Davis et al., 2017). It has shown that microbial communities differed significantly among populations and that the population signature persisted from the tadpole stage through metamorphosis. The initial microbial community strongly influenced the microbiota after metamorphosis (Davis et al., 2017). As, in *A. obstetricans*, most mortalities occur during metamorphosis when the

immune system is non-functional, the tadpole skin microbiota that seeds the metamorphic microbiota is likely of vital importance for the resistance to infection. Moreover, skin microbial communities of tadpoles are less likely to be impacted by dysbiosis. To explore and predict the anti-Bd function of the skin microbiome, we capitalized on the census of genera currently known to potentially inhibit Bd. We did not isolate culturable amphibian skin bacteria and tested for their anti-Bd properties, but rather predicted the putative Bd inhibitory function of the bacteria found in the amphibian skin microbiome, as in several previous studies (Flechas et al., 2019; Goodwin et al., 2022; Jiménez et al., 2022; Nava-González et al., 2021).

We complemented our study with a re-analysis of an already published dataset of amphibian skin microbiome samples collected in 2017, on adults and tadpoles, in the tropical mountains of Taiwan (Schmeller et al., 2022), to test the adaptive microbiome hypothesis in a second geographic range, Asia, where our understanding of the relationship between Bd and amphibian skin microbiome remains relatively limited (Schmeller et al., 2022; Sun et al., 2023), and on the skin microbiomes of adult amphibians as well. The available dataset contained usable information for three species, two anurans (*Hylarana latouchii* and *Nidirana adenopleura*) as well as one caudata (*Hynobius sonani*), spread over 11 sites (of which 6 were positive for Bd and 5 were negative). Unlike the Pyrenees, the Taiwan island is close to the putative region of endemism for Bd (O'Hanlon et al., 2018), and only subclinical infections and no amphibian mass mortality events linked to Bd have been observed (Schmeller et al., 2022). Infection by Bd is widespread across central and northern Taiwan, with only low prevalence and intensity of infection, suggesting that Bd is at endemic equilibrium (Schmeller et al., 2022). We aimed to explore the anti-Bd function of the skin microbiome in this second system in which the hosts and the pathogen have coexisted for longer, but species susceptibility to Bd is unknown.

EXPERIMENTAL PROCEDURES

Study area and sampling design

In 22 Pyrenean Mountain lakes and ponds, we collected samples in 2016, 2017 and 2018. The sites were located along altitudinal gradients (between 1063 and 2522 m above sea level, Table 1), with the longest direct distance between lakes of 173 km from West to East, and 16 km from North to South. Among these

TABLE 1 Number and types of Pyrenean samples used for analysis and the characteristics of the sites from West to East. Ao: *Alytes obstetricans*, Bs: *Bufo spinosus*, Rt: *Rana temporaria*. The Bd status was defined per population.

Site	Altitude (m)	Coordinates	Surface area (ha)	Bd status	Samples
Acherito	1880	42.879; −0.709	7.46	Positive	35 Ao + 5 Bs
Ansabènre	1850	42.888; −0.709	0.21	Positive	25 Ao + 10 Bs + 10 Rt
Lhurs (lake)	1691	42.922; −0.703	3.59	Positive	27 Ao
Lhurs (pond)	1691	42.922; −0.703	0.01	Positive	5 Ao + 10 Rt
Puits (lake)	1880	42.864; −0.633	0.26	Positive	42 Ao
Puits (pond)	1880	42.864; −0.633	0.01	Positive	5 Rt
Arlet	1974	42.840; −0.615	3.46	Positive	16 Ao
Fache Esp.	2522	42.814; −0.246	0.64	Negative	29 Ao + 3 Rt
Grand Fache	2422	42.813; −0.223	0.87	Negative	19 Ao
Embarrat	2180	42.840; −0.190	0.01	Negative	25 Ao + 9 Rt
Vallon	2215	42.838; −0.188	0.05	Negative	33 Ao + 14 Rt
Paradis	1609	42.849; −0.16	0.42	Unclear	25 Ao + 15 Rt
Madamète Ht	2374	42.863; 0.144	0.31	Unclear	28 Rt
Gourg de Rabas	2400	42.852; 0.145	1.33	Negative (cleared Bd)	7 Ao + 4 Rt
Pêcheur	2307	42.866; 0.146	0.59	Negative	5 Rt
Belonguère	1907	42.840; 1.064	0.17	Negative	13 Ao + 22 Rt
Ayès	1714	42.844; 1.065	1.87	Unclear	40 Ao + 15 Rt
Bethmale	1063	42.861; 1.084	2.91	Negative	5 Bs + 5 Rt
Labant	1600	42.775; 1.392	0.46	Negative	10 Rt
Alate	1865	42.777; 1.406	2.13	Negative	15 Rt
Lac Mort	1651	42.765; 1.424	0.86	Negative	2 Bs
Arbu	1737	42.819; 1.437	5.01	Negative	10 Rt
				Total	**341 Ao + 22 Bs + 180 Rt**

TABLE 2 Number and types of Taiwanese samples used for analysis and the characteristics of the sites from West to East. Hl: *Hylarana latouchii*, Hs: *Hynobius sonani*, Na: *Nidirana adenopleura*. The Bd status was defined per population. For the safety of the amphibian populations and species, the coordinates of the sites remain undisclosed.

Site	Altitude (m)	Coordinates	Bd status	Samples
Population 1	169	Undisclosed	Positive	10 Hl adults
Population 2	237	Undisclosed	Negative	1 Na tadpole
Population 3	1060	Undisclosed	Positive	8 Na tadpoles
Population 4	683	Undisclosed	Negative	9 Na tadpoles
Population 5	582	Undisclosed	Positive	2 Hl + 1 Na adult
Population 6	714	Undisclosed	Positive	1 Hl + 2 Na adults + 13 Na tadpoles
Population 7	686	Undisclosed	Negative	4 Hl + 8 Na adults + 10 Na tadpoles
Population 8	808	Undisclosed	Positive	2 Hl adults + 5 Na adults + 8 Na tadpoles
Population 9	3101	Undisclosed	Negative	3 Hs adults
Population 10	2951	Undisclosed	Positive	16 Hs adults
Population 11	2498	Undisclosed	Negative	1 Hs adults
			Total	**19 Hl + 16 Na adults + 49 Na tadpoles + 20 Hs**

sites, 9 had a long history (>10 years) of being infected with Bd, 11 were negative in the long-term, while the disease status of three sites remained ambiguous (Table S1, see also Walker et al., 2010). We visited the sites three times a year (beginning, mid, and end of the growth season of the lake), that is, from the end of June to the beginning of October, as snow typically covers the sites from late October to early June. We sampled skin microbiomes from tadpoles of 3 species: the Common midwife toad *Alytes obstetricans* (342 individuals, 15 sites), the Common frog *Rana temporaria* (186 individuals, 16 sites), and the Spiny common toad *Bufo spinosus* (25 individuals, 4 sites). Most tadpoles of *Alytes obstetricans* were at Gosner stage 25, a few were at stages 26–41. The tadpoles of the other two species were somewhere between Gosner stages 24 and 32. Our goal was to collect microbiome samples from at least five individuals/species/sites/time points when the species was found (not all species were present at all time points). The individuals ($n = 4$–10) were collected by dip-netting around the edge of the lakes, swabbed with sterile dry swabs (MW100, MWE Medical Wire, Corsham, UK) over the entire body (i.e., dorsal, ventral, and tail parts), and released back to site immediately after. We froze the swabs on dry ice (−79°C) in the field, before transferring them to a freezer (−30°C) until DNA extraction. Bd diagnostic samples (target $n = 30$) and skin microbiome samples (target $n = 5$–10) did not necessarily come from the same individuals, which explains the differences in sample size between Tables 1 and S1.

Taiwanese samples were collected and processed in a similar but more opportunistic way so that both adults and tadpoles were sampled in 2017 for skin microbiome and Bd. In the available dataset, we selected groups (species and developmental states) that could be compared between Bd positive and negative sites, resulting in four groups: *Nidirana adenopleura* tadpoles ($n = 49$), *Nidirana adenopleura* adults ($n = 16$), *Hylarana latouchii* adults ($n = 19$), and *Hynobius sonani* adults ($n = 20$). These individuals ($n = 115$) were spread over 11 populations (169–3101 m above sea level, Table 2). The sites included forest populations as well as ponds, and thus surface area of the Taiwanese sites could not be included in the analysis. In 2017, 6/11 populations were infected with Bd. *Nidirana adenopleura* tadpoles and adults had a very low prevalence (2 and 6%) and mean Bd load (<2 GE), while *Hylarana latouchii* and *Hynobius sonani* had higher but moderate prevalence (both 13%) and low mean Bd load (<3 GE) (Table S2).

Skin microbiome sequencing

We extracted DNA from all the 553 Pyrenean swabs using the Macherey-Nagel™ NucleoSpin™ Soil kit (Valencia, CA, USA) and quantified the extraction product using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) to obtain PCR plates with equalized samples (~1 μM DNA). We prepared negative controls from non-used sterile swabs and processed them together with the other samples. We amplified the V3-V4 region of the 16S rRNA gene using Illumina Nextera Index primers and adapters F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG (S-DBact-0341-b-S-17) and R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC (S-D-Bact-0785-a-A-21) which are said to have an amplicon size of 464 bp (Klindworth et al., 2013; Thijs et al., 2017). Our PCR mix (25 μl per well) comprised 12.5 μl MyTaq™ Mix (Bioline), 1 μl of F and R primers (1 μM), 0.5 μl of BSA and 10 μl of sample DNA (~1 μM). We run PCRs as follows: 95°C for 3 min,

35 cycles at 95°C for 30s, 55°C for 30s and 72°C for 30s, and final extension step at 72°C for 5 min. We examined pre-cleanup PCR products on a 1.5% agarose gel. The amplification products were then cleaned with Agencourt AMPure XP (to remove non-target DNA) and a second 1.5% agarose gel electrophoresis was done post-cleanup to ensure the presence of the target DNA. We re-amplified cleaned target DNA for 10 cycles and conducted a second clean-up with Agencourt AMPure XP. This step was followed by a quantification of the indexed target DNA. The indexed DNA samples were pooled at equimolar concentrations, with a starting concentration of 4 nM and a final loading concentration of 14–16 pM as suggested for sequencing on an Illumina MiSeq system, using MiSeq Reagent Kit v2. Demultiplexing and the removal of primer and adapter sequences were performed using Cutadapt v3.4. Additional trimming, formation of contiguous sequences, identification of unique amplicon sequence variants (ASVs), and chimaera removal were performed with the DADA2 v1.20.0 pipeline in R 4.2.0. Taxonomy was assigned using SILVA 138.1. ASVs that were designated as 'unknown' and 'uncultured' by SILVA 138.1 were classified at the lowest identified taxonomic level. Taiwanese samples were processed similarly (see Schmeller et al., 2022 for more details).

We used R4.2.2 with the R packages *phyloseq* (McMurdie & Holmes, 2013), *microbiome* (Lahtie & Shetty, 2012–2019) and *vegan* (Oksanen et al., 2022) to process the data and perform statistical analysis. We removed ASVs that amplified in the negative controls, taxonomically assigned as chloroplast and mitochondria, unclassified at the Domain level, as well as singletons and doubletons. We also removed samples with a low number of reads (Pyrenees: 10 samples <10,000 reads; Taiwan: 11 samples <7,000 reads). We plotted the rarefaction curves to confirm full coverage of the richness of all remaining samples but did not rarefy to avoid losing information. After data cleaning, 543 samples remained in the Pyrenean dataset (Table 1) containing 36,696 ASVs (11,833–185,664 reads): 341 samples of *A. obstetricans* (150 from Bd-positive sites, 126 from Bd-negative sites and 65 in Bd ambiguous ones; 22,629 ASVs), 22 samples of *B. spinosus* (15 in Bd-positive sites, 7 in Bd-negative sites; 4,441 ASVs), and 180 samples of *R. temporaria* (25 in Bd-positive sites, 97 in Bd-negative sites and 58 in Bd ambiguous sites; 23,382 ASVs).

We retained 104 samples in the Taiwanese dataset (Table 2) containing 34,508 ASVs (7,589–632,038 reads): 49 *N. adenopleura* tadpoles (29 in Bd positive sites, 20 in Bd negative; 14,715 ASVs), 16 *N. adenopleura* adults (8 in Bd positive sites, 8 in Bd negative; 8,524 ASVs), 19 *H. latouchii* (13 in Bd positive sites, 6 in Bd negative; 8,362 ASVs), and 20 *H. sonani* (16 in Bd positive sites, 4 in Bd negative; 7,589 ASVs).

Literature review for anti Bd-function

We reviewed the literature to identify 14 published studies reporting 86 genera containing strains of bacteria that were identified as having Bd inhibitory abilities, as well as 4 that were shown to be ineffective ('ns', *Kaistia*, *Leifsonia*, *Limnohabitans*, and *Roseomonas*) (Loyau, 2024). We used this list of genera to compare the putative anti-Bd activity of the amphibian skin microbiome across the host species, and between Bd positive and negative sites. To validate our method (i.e., using genera instead of ASVs), we looked at the correlation between the centred-log transformed relative abundance of the putative inhibitory ASVs of which the sequences were listed in the database of Woodhams et al. (2015). To do so, we used a Linux virtual machine to conduct a standalone nucleotide BLASTN analysis between our sequences and the sequences in the database, with 98 and 100% similarity on nucleic acids and length >200 nucleotides (Johnson et al., 2008; Tao 2010). Our results show high correlation (98% similarity: Pyrenean samples: $n = 543$, $R = 0.76$, $p < 0.001$; Taiwanese samples: $n = 104$, $R = 0.79$, $p < 0.001$; 100% % similarity: Pyrenean samples: $n = 543$, $R = 0.49$, $p < 0.001$; Taiwanese samples: $n = 104$, $R = 0.76$, $p < 0.001$). We thus used the relative abundance of putative Bd-inhibitory genera as a proxy of the inhibitory function of the skin microbiome in this study.

Statistical analysis

We used *vegan* to explore beta-dispersion and beta-diversity between the three Pyrenean amphibian species, and between the four Taiwanese groups, with Bray–Curtis distance and a permutation test within a site (functions betadisper and permutest), followed by a PERMANOVA (999 permutations within a site, function adonis2). When dispersion was significantly different, the conservativeness of the PERMANOVA result was assessed using sample sizes (Anderson & Walsh, 2013), and all results were found conservative unless specified otherwise. We compared number of reads, number of ASVs, Chao1 index and relative abundances with pairwise Mann–Whitney (Wilcoxon rank-sum) tests between species (Pyrenees) or groups (Taiwan).

For the Pyrenean study, we ran non-parametric Kendall correlations with the R package *stats* to explore links between alpha-diversity (number of ASVs and Chao1 index) and anti-Bd function. We used the *ANCOM-BC* package to perform an analysis of the composition of microbiomes with a bias correction using function ancombc2 (clr-transformed abundance, FDR method of *p*-value adjustment) to test genus differential abundance between amphibian species with a

conservative approach; all tests passed the sensitivity check.

We ran general linear models (GLMs) with the *nlme* package to explore the environmental factors explaining the centred-log transformed relative abundance of putative inhibitory genera (glm function with a Gaussian distribution and the identity link function). Explanatory variables included were: host species (*A. obstetricans*, *B. spinosus*, *R. temporaria*), date of sampling and site identity, as well as their two-level and three-level interactions. Due to collinearity between explanatory variables, we ran alternative models by replacing site identity with either longitude, latitude, altitude, size of the surface area, or disease status (positive vs. negative), and compared models with their AICs. For this analysis, we discarded the sites with an ambiguous Bd status, that is, Madamète Haut, (which was infected with Bd in the past, *A. obstetricans* population has now vanished), as well as Ayès and Paradis (which were both initially negative but provided some weak signals of infection—potentially false positives—during the sampling period 2016–2018), leaving us with 420 samples from 19 sites.

We compared dispersion and the overall composition of the skin microbiome between Bd positive and negative sites (discarding the ambiguous sites) with Bray–Curtis distance, a permutation test on dispersion and a PERMANOVA (both within a host species), and confirmed the result with separate PERMANOVAs per amphibian host. Again, we used an *ANCOM-BC* analysis to identify, for each amphibian host, genus differential abundance between Bd positive and negative sites.

We similarly analysed the Taiwan dataset, except that we further separated *N. adenopleura* tadpoles and adults in two groups, because of significantly different skin microbiome communities (betadisper within a site, df = 1, $F = 0.87$, $p = 0.377$; PERMANOVA on Bray–Curtis distance and 999 permutations within a site, df = 2, $F = 8.00$, $R^2 = 0.11$, $p = 0.001$).

RESULTS

Amphibian skin microbiome in the Pyrenees

A. obstetricans and *R. temporaria* had a lower number of reads than *B. spinosus* (Wilcoxon sum-rank test: $W = 2558$, $p = 0.012$; $W = 2733$, $p = 0.004$; *A. obstetricans* vs. *R. temporaria*: W = 33,682; $p = 0.067$). The most abundant inhibitory genera (relative abundance >1%) were *Flavobacterium*, *Acinetobacter*, *Hafnia*, and *Pseudomonas* in *A. obstetricans*, *Flavobacterium*, *Bacillus*, and *Pseudomonas* in *B. spinosus*, and *Acinetobacter*, *Pseudomonas* and *Flavobacterium* in *R. temporaria* (Tables S3–S5, Figure S1).

The beta-dispersion of the whole amphibian microbiome differed between the three Pyrenean species, as *B. spinosus* had lower dispersion than *A. obstetricans* and *R. temporaria* (betadisper within site: df = 1, $F = 54.48$, $p = 0.001$), and their community composition also significantly differed (PERMANOVA within site: df = 2, $F = 13.27$, $R^2 = 0.05$, $p = 0.001$, Figure S2), while their overall alpha-diversity did not (number of ASVs: *A. obstetricans* vs. *B. spinosus*: $W = 3621.5$, $p = 0.787$; *A. obstetricans* vs. *R. temporaria*: $W = 29{,}629$; $p = 0.516$; *B. spinosus* vs. *R. temporaria*: $W = 1990$, $p = 0.971$; Chao1 index: *A. obstetricans* vs. *B. spinosus*: $W = 3614$, $p = 0.775$; *A. obstetricans* vs. *R. temporaria*: $W = 29{,}852$; $p = 0.608$; *B. spinosus* vs. *R. temporaria*: $W = 1999$, $p = 0.942$; Figure S3).

The overall relative abundance of putative inhibitory genera was significantly lower in *A. obstetricans* compared to *B. spinosus* and *R. temporaria* (10.5, 14.1, and 13.2%, respectively, *A. obstetricans* vs. *B. spinosus*: $W = 2634$, $p = 0.019$; *A. obstetricans* vs. *R. temporaria*: $W = 25{,}717$; $p = 0.002$; *B. spinosus* vs. *R. temporaria*: $W = 2198$, $p = 0.401$; Figure 1).

The number of inhibitory ASVs was highly correlated to the total number of ASVs, and the Chao1 index of inhibitory genera was also highly correlated to the overall Chao1 index (Kendall correlations: $Z = 34.8$, $p < 0.001$; $Z = 34.4$, $p < 0.001$). Differential abundance analysis at the genus level between the skin microbiomes of *A. obstetricans* and *B. spinosus* revealed that 77/445 (17.3%) genera were differently associated with one of the two species, including 20 (4.5%) that were putative inhibitory, of which 17/20 (85%) being associated to *B. spinosus* (ANCOM-BC, Figure S4). Comparing *A. obstetricans* and *R. temporaria* showed that 265/456 (58.1%) genera were differently associated, of which 20 (4.4%) were inhibitory, with 18/20 (90%) being associated with *R. temporaria* (Figure S5). Only 59/490 (12.0%) genera were differently associated between *B. spinosus* and *R. temporaria*, 12 (2.4%) were inhibitory, with 11/12 (91.7%) associated with *B. spinosus* (ANCOM-BC, Figure S6). Regarding inhibitory genera, *A. obstetricans* had more abundant *Hafnia* and *Undibacterium* than *B. spinosus* and *R. temporaria*, while *B. spinosus* and *R. temporaria* had more *Brevundimonas*, *Exiguobacterium*, *Pantoea*, *Pedobacter*, *Sanguibacter-Flavimobilis*, *Sphingomonas* and *Variovorax* than *A. obstetricans*.

When exploring the environmental factors explaining the relative abundance of putative inhibitory genera, the host species and the date of sampling were always highly significant and kept in all models (GLMs: all $p < 0.001$). The best model included site identity, a highly significant variable (Table 3). Only altitude did not contribute to explaining the relative abundance of inhibitory genera. Lakes and ponds that were located further east, further south, with a larger surface area, or that were Bd positive had individuals with a higher abundance of inhibitory genera (Table 3, Figure 2A).

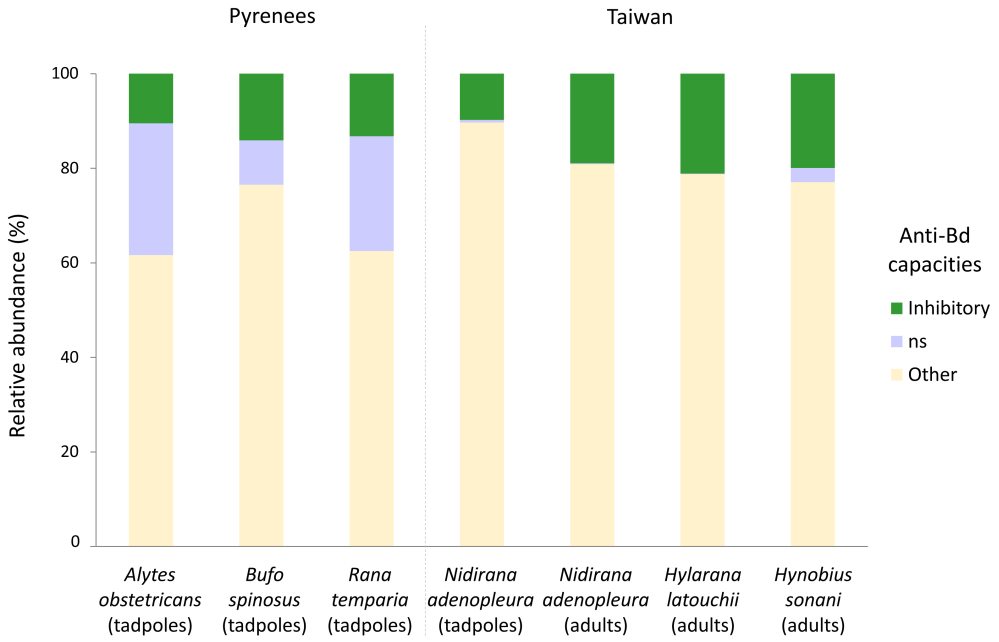

FIGURE 1 Mean relative abundance of the putative inhibitory, non-effective (ns) and 'Other' genera of the skin microbiome of *Alytes obstetricans*, *Bufo spinosus* and *Rana temporaria* (Pyrenees), as well as *Nidirana adenopleura* (tadpoles and adults), *Hylarana latouchii*, and *Hynobius sonani* (Taiwan).

TABLE 3 Comparison of the GLMs based on their AICs for the Pyrenean study (above) and the Taiwanese (below) skin microbiome. (Df: degree of freedom).

Rank	Variable included in the model	LR Chi^2	Df	*P*-value r	AIC of the model
1	Site identity	124.5	18	<0.001	416.7
2	Longitude (W-E)	7.8	1	0.005	538.6
3	Surface area	17.3	1	<0.001	543.8
4	Bd status	10.8	1	0.001	547.6
5	Latitude (N-S)	7.2	1	0.007	554.3
6	Altitude	2.3	1	0.129	556.6
1	Site identity	50.6	8	<0.001	101.6
2	Latitude (N-S)	24.0	1	<0.001	112.1
3	Longitude (W-E)	2.7	1	0.102	114.3
4	Bd status	4.9	1	0.027	126.1
5	Altitude	1.9	1	0.164	137.6

The overall beta-dispersion of the skin microbiome was significantly lower in Bd-positive populations compared to Bd-negative ones (betadisper within host species: df = 1, F = 54.48, $p = 0.001$), which held true in *A. obstetricans* (df = 1, F = 17.83, p = 0.001) and *R. temporaria* (df = 1, F = 37.35, p = 0.001) but was not different in *B. spinosus* (df = 1, F = 1.40, $p = 0.239$). The overall community composition also differed according to the Bd status of the site (PERMANOVA within host species: df = 1, F = 20.29, $R^2 = 0.05$, p = 0.001, Figure S7), and verified for host species separately (all p-values <0.05, and all PERMANOVAs conservative except for *A. obstetricans*). Bd positive populations had a higher number of ASVs and higher Chao1 index compared to negative ones, both overall (W = 10,927; $p < 0.001$; $W = 10{,}815$; $p < 0.001$, respectively, Figure S8) and for each amphibian host tested separately (*A. obstetricans*: $W = 3899.5$, $p < 0.001$; $W = 3877$, $p < 0.001$; *B. spinosus*: $W = 20$, $p = 0.021$; $W = 20$, $p = 0.021$; *R. temporaria*: $W = 403$, $p < 0.001$; $W = 397$, $p < 0.001$). The ANCOM-BC revealed that, in *A. obstetricans*, 221/467 (47.3%) genera were differently associated with Bd negative versus positive sites, of which 22 (10.0%) were putative inhibitory, including 14/22 (63.6%) associated to negative sites, while 8/22 (36.4%) were associated to Bd positive sites. In *B.*

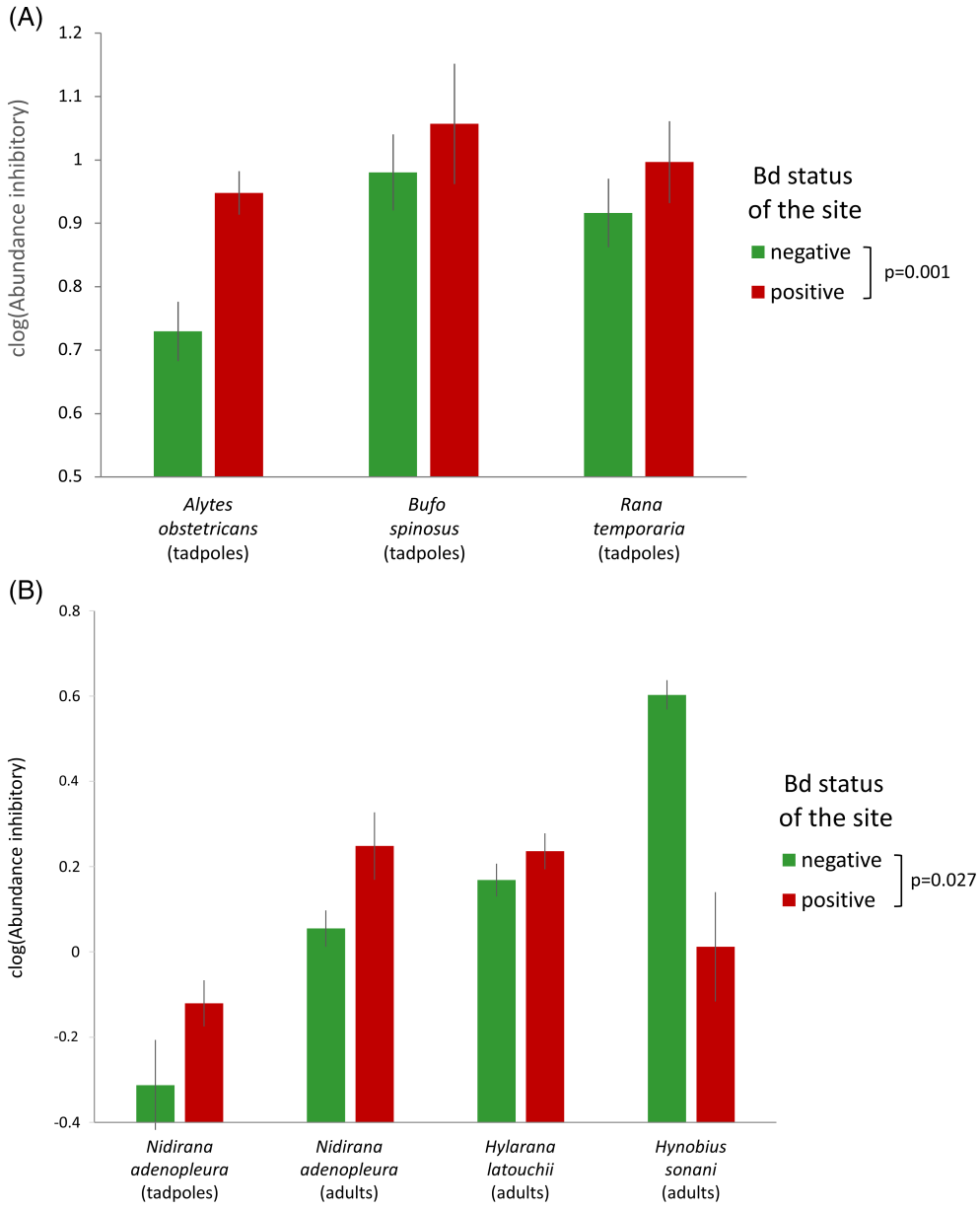

FIGURE 2 Centered-log-mean relative abundance of the putative inhibitory genera of the skin microbiome of (A) *Alytes obstetricans*, *Bufo spinosus* and *Rana temporaria* for the Bd negative and Bd positive sites (Pyrenees) and of (B) *Nidirana adenopleura* tadpoles and adults, *Hylarana latouchii* (adults) and *Hynobiusana sonani* (adults) for the Bd negative and Bd positive sites (Taiwan). The values represented are the mean ± standard error of the mean.

spinosus, only 36/439 (8.2%) genera were differently associated according to the Bd status, including 7/36 (19.4%) inhibitory, of them only 2/7 (28.6%) were associated with Bd positive sites. In *R. temporaria*, 85/512 (16.6%) genera showed differences, with 10/85 (11.8%) being inhibitory, most of which (9/10, 90%) being associated with Bd positive sites. Bd infection status was linked to increased abundance of *Bacillus* and *Lysinibacillus* in all three amphibian hosts, as well as abundance of *Arthrobacter*, *Aeromonas*, *Flavobacterium* and *Exiguobacterium* in both *A. obstetricans* and *R. temporaria* (ANCOM-BC, Figure 3).

Amphibian skin microbiome in Taiwan

There was no difference in the number of reads between Taiwanese groups (Wilcoxon sum-rank test: all *p*-values >0.05). The most abundant inhibitory genera (relative abundance >1%) were *Pseudomonas* and *Stenotrophomonas* in *N. adenopleura* tadpoles, *Pseudomonas*, *Acinetobacter*, *Sphingomonas*, *Brevundimonas*, and *Deinococcus* in *N. adenopleura* adults, *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, and *Flavobacterium* in *H. latouchii*, and *Flavobacterium* and *Pseudomonas* in *H. sonani* (Tables S6–S9, Figure S9).

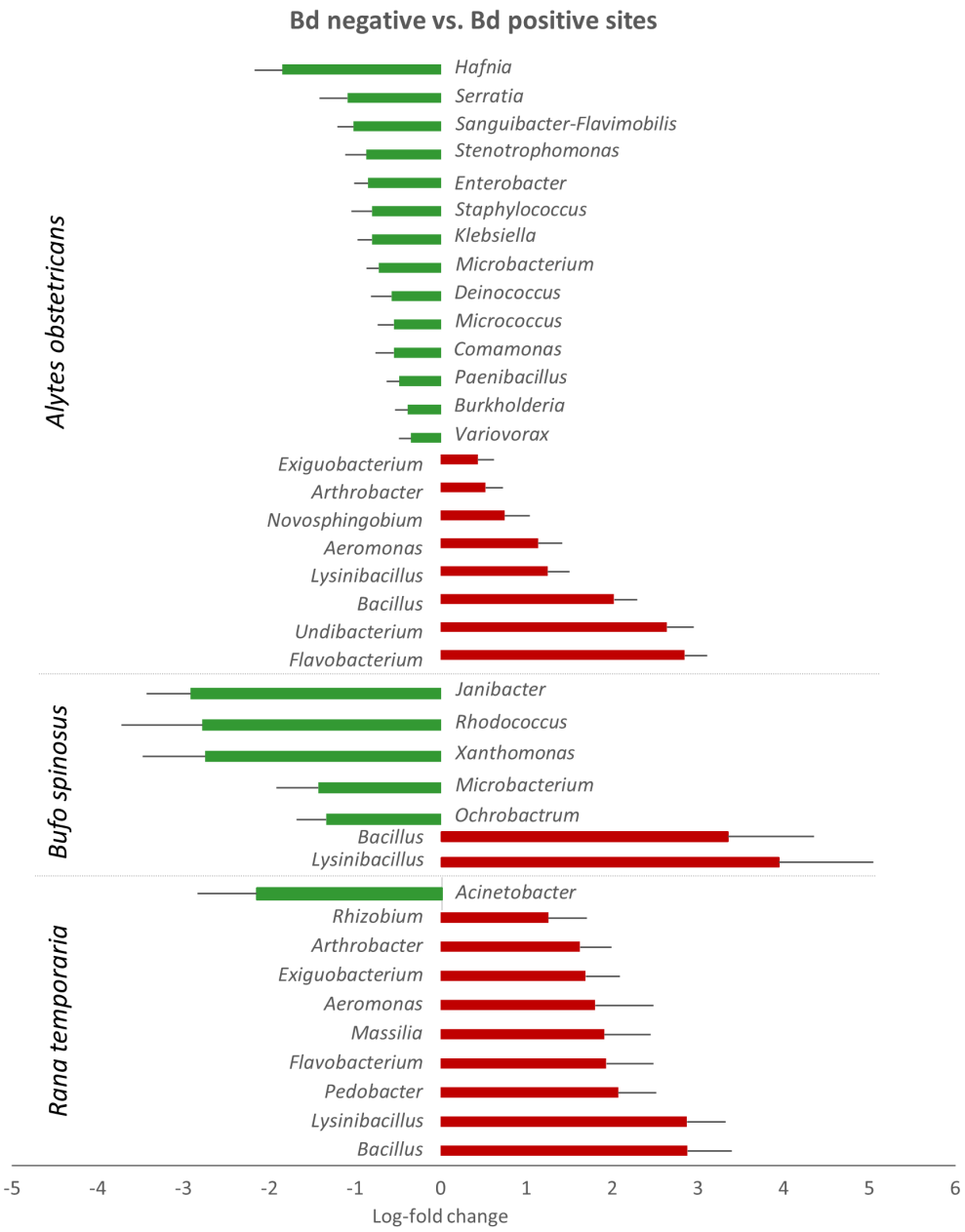

FIGURE 3 Putative inhibitory genera that are differently associated with Bd negative (green) and positive sites (red) in the Pyrenees, for *Alytes obstetricans*, *Bufo spinosus*, and *Rana temporaria* as revealed by an analysis of compositions of microbiomes with bias correction (ANCOM-BC).

The whole skin microbiome showed a significantly different composition and dispersion between the groups (betadisper: df = 1, $F = 0.87$, $p = 0.377$; PERMANOVA: df = 3, $F = 6.32$, $R^2 = 0.16$, $p = 0.001$; Figure S10), including between *N. adenopleura* tadpoles and adults (betadisper: df = 3, $F = 1.53$, $p = 0.234$; PERMANOVA: df = 2, $F = 8.00$, $R^2 = 0.11$, $p = 0.001$). *N. adenopleura* tadpoles had higher number of ASVs and Chao1 index than *N. adenopleura* adults ($W = 151.5$, $p < 0.001$; $W = 151.0$, $p < 0.001$; Figure S11) and *H. sonani* had also higher alpha-diversity indices than *H. latouchii* (number of ASVs: $W = 109$, $p = 0.022$; Chao1: $W = 106$, $p = 0.018$; Figure S11). *N. adenopleura* tadpoles had lower overall relative abundance of putative inhibitory genera compared to the other groups (*N. adenopleura* tadpole vs. adults: $W = 597.0$, $p = 0.001$; *N. adenopleura* tadpole vs. *H. latouchii*: $W = 844.0$, $p < 0.001$; *N. adenopleura* tadpole vs. *H. sonani*: $W = 650.0$, $p = 0.034$; Figure 1). In GLMs, the group (considering species and developmental stage) was always significant and kept in all models (all $p < 0.02$). Similarly, the date of sampling was always included in the model to improve the AIC, except for the model with only site identity (and group),

due to collinearity. As for the Pyrenean GLMs, the best model included site identity, a highly significant variable (Table 3). The latitude and Bd status of the site were both significant, with all anuran groups having a higher relative abundance of Bd inhibitory genera in Bd-positive sites while showing the opposite pattern in the caudata *H. sonani* (Table 3, Figure 2B). The community composition of the skin microbiome significantly differed between Bd positive and negative populations when all groups were considered together (betadisper: df = 1, $F = 0.46$, $p = 0.519$; PERMANOVA within host groups: df = 1, $F = 3.24$, $R^2 = 0.03$, $p = 0.001$; Figure S12), and this difference remained when comparing within a host group (all betadispers p-value >0.05; all PERMANOVA p-values <0.02), except for *H. latouchii* in which individuals of positive sites had a higher dispersion (betadisper: df = 1, $F = 24.99$, $p = 0.001$). The alpha diversity was not significantly different between Bd positive and negative populations when groups were compared altogether ($W = 965$, $p = 0.077$; $W = 970$, $p = 0.083$, Figure S13). When groups were considered separately, only *H. sonani* had a higher number of ASVs and Chao1 index in positive sites, although close to the significance threshold (*N. adenopleura* tadpoles: $W = 232$, $p = 0.245$ and $W = 234$, $p = 0.262$; *N. adenopleura* adults: both

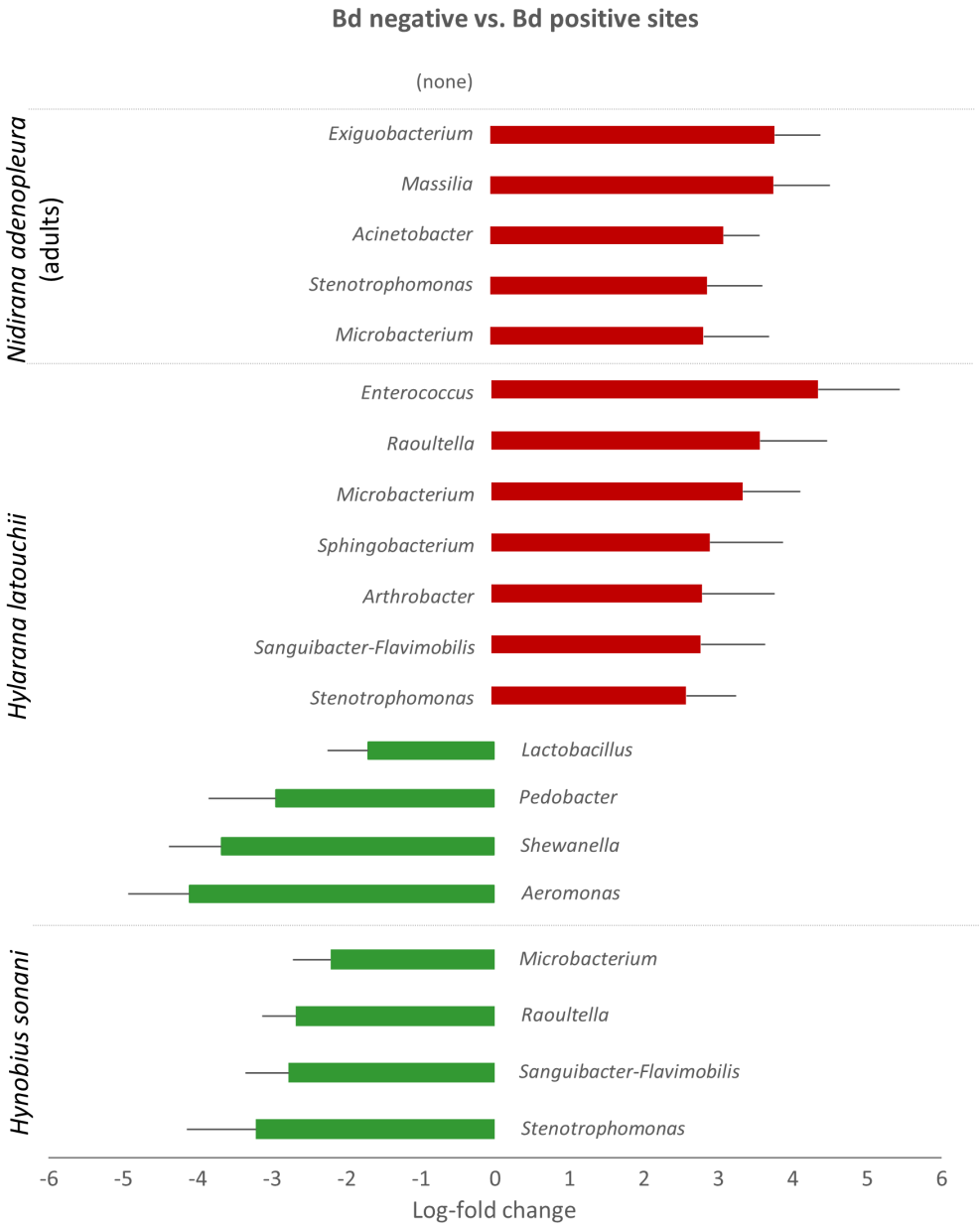

FIGURE 4 Putative inhibitory genera that are differently associated with Bd negative (green) and positive sites (red) in Taiwan, for *Nidirana adenopleura* adults, *Hylarana latouchii* adults, and *Hynobius sonani* adults as revealed by an analysis of compositions of microbiomes with bias correction (ANCOM-BC). The *Nidirana adenopleura* tadpoles did not have any different putative inhibitory genera.

$W = 23$, $p = 0.382$; *H. latouchii*: $W = 21$, $p = 0.411$ and $W = 23$, $p = 0.530$; *H. sonani*: both $W = 11.0$, $p = 0.049$, respectively). The ANCOM-BC revealed that, in *N. adenopleura* tadpoles, 18/874 (2.1%) genera were differently associated regarding the Bd status of the site, all of them associated with Bd negative sites, and none of them recognized for anti-Bd capacities. In *N. adenopleura* adults, 54/724 (7.5%) genera showed a significant difference, of which 27/54 (50.0%) were associated with Bd negative sites (and none of them being putative inhibitory), while in the other half, 5/27 (18.5%) were inhibitory genera. *Hylarana latouchii* had 79/807 (9.8%) genera differently associated: 54/79 (68.4%) were associated with Bd negative sites, of which 4/54 (7.4%) were inhibitory, while of the 25/54 (46.0%) associated to Bd positive sites, 7/25 (28.0%) were inhibitory. Finally, in the caudata *H. sonani*, only 22/553 (4.0%) exhibited a significant difference, with 17/22 (77.3%) associated with negative sites (4/17, 23.5% being inhibitory), and 5/22 (22.7%) associated to the presence of Bd (none of them being putative inhibitory). In the Taiwanese skin microbiome, the genera involved in these differences varied strongly between the groups. *Stenotrophomonas* and *Microbacterium* were associated with the presence of Bd in *N. adenopleura* adults and *H. latouchii*, while they were both associated with the absence of Bd in *H. sonani* (Figure 4).

DISCUSSION

We analysed the commensal skin microbiome of tadpoles of three amphibian species in the French Pyrenees sharing the same habitat (high mountain lakes), as well as three amphibian species in Taiwan. Our results confirm the role played by the amphibian host species in shaping the microbial composition of the skin, in accordance with previous studies (e.g., Belden et al., 2015; Kueneman et al., 2014). We particularly focused on genera known to inhibit the proliferation of the fungal pathogen Bd, a globally emerged pathogen driving populations and species extinctions worldwide. Interestingly, in the Pyrenees, the susceptibility of the host species to the pathogen was reflected in the skin microbiome. While the overall alpha diversity did not differ between the three species, *A. obstetricans*, the species most susceptible to Bd, had a lower amount of putative anti-Bd bacteria than the less susceptible *B. spinosus* and *R. temporaria*. The comparison of two Peruvian marsupial frogs, *Gastrotheca excubitor* and *G. nebulanastes*, also revealed that the Bd-resistant species had more cultivable anti-Bd skin bacteria than the susceptible one, both in number and proportion (Burkart et al., 2017).

It is more and more accepted that the amphibian skin communities are selected rather than randomly assembled from the aquatic environment (Walke et al., 2014). However, what mediates the recruitment of protective microbes remains unclear and may be linked to host physiology or environmental factors. The cutaneous granular glands of the amphibian hosts may secrete different toxins and antimicrobial peptides which are likely to modify the immediate environment of the skin microbial community and contribute to disease resistance (Davis et al., 2017; Flechas et al., 2019). Differences in the availability of Bd-inhibitory bacteria at the time of egg-hatching may also be due to *A. obstetricans*, *B. spinosus* and *R. temporaria* showing different life history traits and reproductive phenology. *R. temporaria* lays eggs early in the season, followed by *B. spinosus*, and both finish the development from egg to froglet within the same year, even in mountain lakes. *A. obstetricans* lay eggs usually in mid-summer, and tadpoles do not finish their metamorphosis in the same year and can hibernate for up to several years in the lakes. Recruitment of microorganisms may therefore differ in these three host species and may translate into differences in host susceptibility to Bd and other pathogens such as ranaviruses. Unfortunately, we could not assess inter-species differences regarding Bd susceptibility in Taiwanese hosts, as Bd prevalence and infection load were low and amphibian hosts are likely to have coexisted with Bd for a while (Schmeller et al., 2022).

We were able to compare the skin microbiome of larvae and adults only of Taiwanese *N. adenopleura* and we found that their microbiomes varied strongly in composition. This is congruent with a reshaping of the microbial communities during metamorphosis, as already shown for the Colorado boreal toad *Anaxyrus boreas* (Kueneman et al., 2016; Prest et al., 2018). Although *N. adenopleura* adults had less diverse microbiomes than tadpoles, they had higher relative abundances of anti-Bd bacteria, suggesting that metamorphosis may facilitate the proliferation, and maybe the selection of protective bacteria with host susceptibility to Bd increases. However, more work is needed to elucidate these processes.

In the populations we studied, both in the Pyrenees and in Taiwan, we found populational differences in the skin microbiome. We also found a difference in the microbial assemblage when Bd infects some members of the community, which translated into increased relative abundances of anti-Bd bacteria in all anuran species. In the Taiwanese salamander *H. sonani*, we observed the opposite pattern. *H. sonani* was the only salamander species in our dataset, so we have little data to explain the observed pattern. *H. sonani* was also the only species we sampled in a terrestrial habitat, which might have impacted on its skin microbiome. Sun and collaborators also found a different pattern in putative inhibitory bacteria associated with Bd presence/absence in a terrestrial species compared to an

aquatic one in Asian anuran hosts (Sun et al., 2023). Further work would be needed to see if the pattern in *H. sonani* can be observed in other salamander species in Taiwan or if it is an effect of the environment.

Our results support the adaptive microbiome hypothesis which states that environmental stressors can lead to an alternate state of the microbiome better suited to the new conditions that persist as long as the stressor remains or host death occurs (Woodhams et al., 2023). As such, pathogen exposure can lead to increased anti-pathogen function that is beneficial to the host immediately and in case of secondary exposure (Woodhams et al., 2023). Previous studies in anuran and caudata hosts have investigated the link between the abundance of putative Bd-inhibitory taxa and Bd infection; all have found a negative correlation, suggesting that inhibitory taxa may be able to protect hosts against Bd or maintain infection at low intensity (Flechas et al., 2019; Goodwin et al., 2022; Jiménez et al., 2022; Kruger, 2020; Nava-González et al., 2021; Siomko et al., 2023). The next step is now to investigate whether these putative inhibitory microbes can inhibit Bd in natural conditions and confer a form of protection, as this ability may vary with environmental conditions (Bernardo-Cravo et al., 2020; Woodhams et al., 2018).

According to the adaptive microbiome hypothesis, three scenarios are possible following primary exposure to a pathogen: (1) the adaptive microbiome returns to a state close to the initial state, and the hosts recover, with a moderate increase in dispersion and richness; (2) the adaptive microbiome persists over time, allowing the hosts to survive due to microbial rescue, resulting in decreased dispersion and richness; or (3) dysbiosis occurs, the hosts become moribund, and this is evidenced by a significant increase in dispersion and richness, aligning with the Anna Karenina principle (Woodhams et al., 2023; Zaneveld et al., 2017).

In Taiwanese anuran hosts, although the relative abundance of protective microbes increased in the presence of the pathogen, there was globally no difference in dispersion and richness in the presence and absence of Bd, which may be explained by a longer history of co-evolution of amphibian hosts with the pathogen (Schmeller et al., 2022). In contrast, Bd is an emerging pathogen in the Pyrenees (Walker et al., 2010), and for all three Pyrenean hosts we observed a decreased dispersion and an increased richness compared to uninfected populations, showing a link between the presence of the pathogen and a correlation with the selection of protective bacteria. Given that *A. obstetricans*, *B. spinosus* and *R. temporaria* have different susceptibility to Bd infection and exhibit a different chytridiomycosis outcome, we expected the three hosts to follow a different pattern. Our results may be explained by the fact that tadpoles usually exhibit minimal –at least localized– clinical signs of infection compared to individuals undergoing metamorphosis. Interestingly, a previous study compared the skin microbiome of *A. obstetricans* metamorphs belonging to Pyrenean populations that were either declining (epizootic) or persistent (enzootic) in the presence of Bd (Bates et al., 2018). Bates and colleagues used Operational Taxonomic Units (OTUs), which are clusters of similar sequence reads (equivalent to ASVs), typically groups based on a threshold of sequence similarity to approximate species-level classification. OTU-alpha-diversity was reduced in epizootic populations (Bates et al., 2018). We examined the OTUs differently associated with the epizootic and enzootic populations of the study of Bates and colleagues. We found that only 3.3% (2/61) of OTUs associated with enzootic populations belonged to a putative inhibitory genus (i.e., *Novosphingobium* and *Sphingomonas*), while, in epizootic populations, OTUs with anti-Bd properties were more numerous (16/25, 64.0%) and diverse (i.e., they belonged to 10 genera) (Bates et al., 2018). These numbers suggest an adaptation to Bd presence and chytridiomycosis, yet these taxa failed to promote disease resistance in these populations. Experimental work is now needed to better understand how the relative abundance of putative inhibitory microbes can translate into improved resistance to chytridiomycosis, as many factors are known to modulate interactions between the host microbiome and disease outcome (Bernardo-Cravo et al., 2020).

Understanding the functional roles of microbes is a key issue in microbial ecology. One method to infer the inhibitory properties of the amphibian skin microbiomes involves culturing, testing and sequencing bacteria isolates, which has the disadvantage of being biased in favour of culturable bacteria. If these bacteria represent only a small proportion of the bacterial assemblage, most of the dominant taxa are likely to be found in cultures (Walke et al., 2015). To capture a larger proportion of the community, including dormant bacteria, we used high-throughput sequencing coupled with published data from in vitro culture of isolates to predict the putative anti-Bd properties at the genera level. We found a strong correlation between the relative abundance of putative inhibitory bacteria genera estimated by our method and the relative abundance estimated by sequence matching of a large database of sequenced isolates (Woodhams et al., 2015). We emphasize that it is only a predictive method, less precise than culture-dependent and '-omics' methods, however, it is an essential first step in inferring the functional role of amphibian symbiotic microbiomes in anti-Bd immunity, paving the way for the isolation and culture of targeted anti-Bd bacteria.

The scientific community is now documenting examples of the recovery of amphibian populations following the Bd epidemic wave (e.g., Hollanders et al., 2023). In Panama, the virulence of the fungal pathogen has not diminished and recovery may be linked to improved skin defences since Bd's emergence (Voyles

et al., 2018). Co-evolution of host defences with the presence of a pathogen takes time before adaptation can occur. In this arms race, commensal microbial assemblages able to confer a form of protection, even imperfect, could reduce pathogen load and may buy time to develop other forms of immunity over time.

AUTHOR CONTRIBUTIONS

Adeline Loyau: Conceptualization; investigation; funding acquisition; writing – original draft; methodology; formal analysis. **Rayan Bouchali:** Validation; methodology; writing – review and editing. **Hugo Sentenac:** Methodology; validation; writing – review and editing. **Dirk S. Schmeller:** Conceptualization; investigation; funding acquisition; validation; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All sequences and metadata information are openly available in the ENA database under the accession number: PRJEB46609 (ERP130803): https://www.ebi.ac.uk/ena/browser/view/PRJEB46609.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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