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HOST MICROBE INTERACTIONS

Probiotics Modulate a Novel Amphibian Skin Defense Peptide That Is Antifungal and Facilitates Growth of Antifungal Bacteria

Douglas C. Woodhams[1] · Louise A. Rollins-Smith[2,3] · Laura K. Reinert[2] · Briana A. Lam[4] · Reid N. Harris[4] · Cheryl J. Briggs[5] · Vance T. Vredenburg[6] · Bhumi T. Patel[1] · Richard M. Caprioli[7] · Pierre Chaurand[8] · Peter Hunziker[9] · Laurent Bigler[10]

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Abstract

Probiotics can ameliorate diseases of humans and wildlife, but the mechanisms remain unclear. Host responses to interventions that change their microbiota are largely uncharacterized. We applied a consortium of four natural antifungal bacteria to the skin of endangered Sierra Nevada yellow-legged frogs, *Rana sierrae*, before experimental exposure to the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*). The probiotic microbes did not persist, nor did they protect hosts, and skin peptide sampling indicated immune modulation. We characterized a novel skin defense peptide brevinin-1Ma (FLPILAGLAANLVPKLICSITKKC) that was downregulated by the probiotic treatment. Brevinin-1Ma was tested against a range of amphibian skin cultures and found to inhibit growth of fungal pathogens *Bd* and *B. salamandrivorans*, but enhanced the growth of probiotic bacteria including *Janthinobacterium lividum*, *Chryseobacterium ureilyticum*, *Serratia grimesii*, and *Pseudomonas* sp. While commonly thought of as antimicrobial peptides, here brevinin-1Ma showed promicrobial function, facilitating microbial growth. Thus, skin exposure to probiotic bacterial cultures induced a shift in skin defense peptide profiles that appeared to act as an immune response functioning to regulate the microbiome. In addition to direct microbial antagonism, probiotic-host interactions may be a critical mechanism affecting disease resistance.

Keywords Amphibian · Antimicrobial peptide · Chytridiomycosis · Disease ecology · Immune regulation · Immunomodulation · Microbiota · Promicrobial

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✉ Douglas C. Woodhams
douglas.woodhams@umb.edu; dwoodhams@gmail.com

Louise A. Rollins-Smith
louise.rollins-smith@vanderbilt.edu

Laura K. Reinert
laura.reinert@vumc.org

Briana A. Lam
briana.lam@jmu.edu

Reid N. Harris
harrisrn@jmu.edu

Cheryl J. Briggs
cherie.briggs@lifesci.ucsb.edu

Vance T. Vredenburg
vancev@sfsu.edu

Bhumi T. Patel
bhumi.patel001@umb.edu

Richard M. Caprioli
richard.m.caprioli@vanderbilt.edu

Pierre Chaurand
pierre.chaurand@umontreal.ca

Peter Hunziker
peter.hunziker@uzh.ch

Laurent Bigler
laurent.bigler@chem.uzh.ch

Extended author information available on the last page of the article

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Introduction

Microbiota are significant in the ecology of disease by shaping host immunity and affecting the context for infection. Important components of host mucosal surfaces such as the skin and gastrointestinal tract are defense peptides, commonly termed antimicrobial peptides (AMPs), with significant immune functions across a wide range of host organisms [1–4]. Because of the limited potency of some amphibian skin defense peptides, Conlon [5] hypothesized that peptides may play a role secondary to the microbiota in terms of pathogen defense. Recent studies have indicated natural selection of both skin microbiota and defense peptides in the recovery of amphibian populations from disease emergence [6, 7]. Here, we extend Conlon's hypothesis and suggest that expression of defense peptides on amphibian skin is in part a response to microbiota, and defense peptides in some species of amphibians help to maintain the composition of the microbiome.

In insects, induction of defensive peptides occurred late in the immune response [8], and recent studies in mammals have shown that peptides are inducible by factors ranging from metals and amino acids to vitamin D and sunlight [9]. Indeed, sodium butyrate can increase AMP expression [10, 11], and butyrate and other metabolites have important roles in microbiome succession, and host recovery from low-diversity dysbiosis [12]. In addition, hosts may regulate the expression of AMPs under different environmental conditions, including stress [13], seasonal variation [14–16], or conditions of microbial exposure [17]. Some microbes have developed mechanisms to evade host defenses including deactivation of AMPs [18, 19]. Regardless of the potential for evolution of resistance [20, 21], research continues to focus on the therapeutic potential of AMPs, particularly in light of antibiotic resistance [9, 22–24].

Here, we test the amphibian host AMP response to interventions of the microbial environment. A probiotic proof-of-concept study showed that addition of a bacterium can increase survival of frogs exposed to the fungal skin pathogen, *Batrachochytrium dendrobatidis* (*Bd*) [25]. In that study, the bacterium used for therapy was *Janthinobacterium lividum* which produces the potent antifungal compound violacein [26]. Thus, one avenue for effective antifungal biotherapy is to emphasize microbial antagonism, or direct competition between pathogenic and non-pathogenic microbes [4, 27]. This mechanism has been the focus for research on probiotic therapy for management of amphibian disease [28, 29]. The effect of biotherapy on host immunity is a pathway not previously examined, and an alternative mechanism of probiotic effect includes activation of host AMP defenses.

Three AMPs were previously described from mountain yellow-legged frogs, *Rana muscosa* (now split into *R. muscosa* and *R. sierrae*) [30]: ranatuerin-2Ma, ranatuerin-2Mb, and temporin-1M. The two AMPs in the ranatuerin-2 family and one AMP from the temporin-1 family were described with similar structures to peptides in related frog species. Other, less abundant peptides were present in skin secretions, including a peptide of approximate mass 2524.5 Da, but when these HPLC fractions were tested against *Staphylococcus aureus* and *Escherichia coli*, no potent growth-inhibitory activity was indicated, and the peptides were not further described [30]. A central aim of our study was to determine how probiotic therapy affects host AMPs, and whether a novel defense peptide has both "promicrobial" (facilitating microbial growth) and antimicrobial properties. This study explores mechanisms underlying probiotics and may lead to the development of therapies to enhance or stimulate host immunity against pathogens including the emerging chytrid fungi devastating amphibian populations [31].

Methods

Animal Husbandry

Scientific research and collecting permits were granted from the United States Department of the Interior, National Park Service. All experiments conformed to the ethical and animal care guidelines and were approved by the James Madison University Institutional Animal Care and Use Committee. *Rana sierrae* eggs were collected from multiple egg masses during the breeding season of June 2006 in the Sixty Lake Basin of the Sierra Nevada Mountains, California. The eggs were salvaged from a pool that would later dry. After raising the tadpoles at the University of California at Berkeley, metamorphs were shipped to James Madison University for an experiment that started in October 2007. Each of the 60 individual froglets (mean mass ± SD = 2.52 ± 0.68 g) was housed individually in a bleached and autoclaved (sterilized) plastic enclosure containing approximately 200 mL sterile Provosoli medium (artificial pond water [32]). The recipe for Provosoli medium (pH 7.0, autoclave or filter-sterilize) is 2 mL of each salt solution added to 1 L deionized water: $NaNO_3$ (6.25 g in 250 mL diH_2O), $MgSO_4 \cdot 7H_2O$ (5.0 g in 250 mL diH_2O), $CaCl_2 \cdot 2H_2O$ (3.3 g in 250 mL diH_2O), K_2HPO_4 (0.75 g in 250 mL diH_2O), KCl (6.25 g in 250 mL diH_2O), and KH_2PO_4 (0.75 g in 250 mL diH_2O). Each enclosure was randomly assigned to one of five treatments placed randomly on a metal rack in a temperature-controlled room set at 17 °C with a 12-h light cycle.

Frogs were fed approximately five crickets weekly. Cages were sterilized and freshwater was added twice weekly.

Experimental Design

We performed a replicated, randomized experiment with five groups of *R. sierrae*: (1) symbionts reduced, probiotics, *Bd*; (2) symbionts reduced, *Bd*; (3) symbionts reduced, probiotics; (4) symbionts reduced control; and (5) control (Table 1). Based on the assumption that initially reduced bacterial abundance and reduced microbial-community interactions would maximize the probability of establishing a new protective skin microbiota upon application of probiotics, we pretreated four of the five treatment groups. Thus, 50 froglets were briefly washed in a mild antibacterial detergent (0.12% triclosan) and given a 30-s rinse in 3% hydrogen peroxide immediately followed by a rinse with sterile Provosoli medium. Pilot experiments showed that this treatment did not harm the frogs, reduced culturable bacterial diversity, and was thought to standardize initial frog skin condition, as shown in previous studies [25, 33].

One and five days after the pretreatment, probiotic bacteria were added to 26 of the frogs, and the remaining frogs were sham-exposed to sterile Provosoli media only. Four bacterial isolates were chosen based on a previous study that identified *Bd* growth inhibition in co-culture assays [34]. The isolates included approximately 27×10^9 *Pseudomonas fluorescens*, 15×10^9 *Pedobacter cryoconitis*, 13×10^9 *Chryseobacterium* sp., and 11×10^8 *Iodobacter* sp. Cultures were grown in nutrient broth for 2 days at room temperature with continuous stirring. Cultures were then centrifuged at 4500*g* for 10 min. The supernatant was discarded and the pellets re-suspended in sterile Provosoli medium. This washing step was then repeated. Finally, 1 mL of each bacterium was added to 21 mL sterile Provosoli medium and the froglet in a 120-mL plastic dish for a 2-h exposure with shaking every 30 min. Microbial communities were allowed to establish on the skin for approximately 2 weeks before exposure to *Bd*.

On days 13 and 15 after pretreatment, 30 frogs were exposed to *Bd* and the rest were sham-exposed to sterile media.

Table 1 Experimental design for *Rana sierrae* treatments

Group	*N*	Microbiota reduction	Probiotic addition	*Bd* exposure
1	16	Yes	Yes	Yes
2	14	Yes	No	Yes
3*	10	Yes	Yes	No
4*	10	Yes	No	No
5	10	No	No	No

*AMPs collected on day 65 of the experiment

The *Bd* isolate was obtained from a *R. muscosa* at Conness pond (Tate Tunstall, TST 75). Frogs were exposed to a solution containing approximately 35×10^4 *Bd* zoospores (or sterile wash) in 25 mL Provosoli medium for 24 h.

Frog Experiment Measurements and Analyses

Kaplan-Meir survival analysis was performed on censored survival data [35]. The slope of mass change throughout the experiment was compared among treatments with an ANOVA using weekly mass measurements. Statistics were performed in IBM SPSS Statistics v19. An analysis of *Bd* infection load was deemed unnecessary for this experiment given the high mortality of *Bd*-exposed frogs compared with those not exposed to *Bd* in this and other experiments [25, 36]. To examine the relative persistence of the probiotic bacteria on the skin of frogs, a culture-independent analysis of the microbial community was performed using denaturing gradient gel electrophoresis (DGGE). For this analysis, skin swabs were taken from ten probiotic-treated and five control frogs upon completion of the experiment 65 days after the final exposure to *Bd*. DNA was extracted and 16S rDNA genes were amplified as previously described [37, 38]. Skin peptides were collected on day 65 from ten control frogs (treatment 3) and ten probiotic-treated frogs (treatment 4) to determine if treatment interacted with this host immune defense. Induction of peptide secretion was performed according to Woodhams et al. [34] using 10 nmol/g body mass norepinephrine-bitartrate (Sigma, St. Louis, MO) injected subcutaneously on the dorsal surface. Peptide mixtures were immediately acidified to prevent proteolytic degradation and were partially purified and concentrated over C-18 Sep-Pak cartridges (Waters Corp., Milford, MA). Peptides were then quantified by Micro BCA assay (Pierce, Rockford, IL) using bradykinin (FW 1060.2, Sigma) as a small peptide standard, and tested for their ability to inhibit the growth of *Bd* as previously described [34, 39]. Because peptides were induced from granular glands, samples do not represent the peptides constitutively expressed on the skin, but rather those synthesized and stored within the glands, as well as those already present and washed from the skin surface.

Mass Spectrometry to Compare Peptide Profiles Between Treatments and to Determine Structure of Novel Peptide, Brevinin-1Ma

Skin peptide mixtures were analyzed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) and high-performance liquid chromatography (HPLC) MS. Detailed methods for MALDI-MS, HPLC-MS fractionation, and Edman degradation can be found in Supplementary Materials. Relative

abundance of each peptide was compared between treatments, and peptide relative abundance determined by HPLC-MS was tested for correlation with relative abundance determined by MALDI-MS. The amino acid sequence of brevinin-1Ma was confirmed by MS/MS and Edman degradation as described in Supplementary Materials.

AMP Database Alignment

The brevinin-1Ma sequence was aligned against 3053 antimicrobial peptide sequences in the antimicrobial peptide database (http://aps.unmc.edu/AP/, accessed January 21, 2019 [40]). The closest match is reported.

Synthesis of Brevinin-1Ma and Testing for Effects on Microbial Growth

Brevinin-1Ma (FLPILAGLAANLVPKLICSITKKC) and a similar artificial peptide that substituted four isoleucines for leucines (FLPIIAGIAANIVPKIICSITKKC) were synthesized commercially (China Peptides Co., Ltd., Shanghai, China) and tested for their ability to inhibit the growth of *Bd* zoospores. As previously described [39], we measured the growth inhibition caused by peptide concentrations ranging from 1.56 to 200 μM and determined the minimal inhibitory concentration (MIC), or lowest concentration that completely inhibited *Bd* growth, for both peptides. The peptides were tested against both the type strain JEL 197 and a *R. muscosa* isolate of *Bd*, CJB 215.

In addition to *Bd*, we tested for growth effects of brevinin-1Ma on another emerging fungal pathogen of amphibians, *B. salamandrivorans* (*Bsal*), as well as six bacterial and one fungal isolate from amphibian skin. These included *Penicillium* sp., *Chryseobacterium indologenes*, *C. ureilyticum*, *Serratia grimesii*, *Pseudomonas* sp., *Janthinobacterium lividum*, and *Rhodococcus fasciens.* The skin microbes were chosen because they represent a range of common anti-*Bd* symbionts found on amphibian skin [27, 34, 41]. We tested for both growth inhibition and facilitation by the peptide at concentrations of 12.5 and 50 μM.

Results

Survival

Overall, there was a significant effect of treatment on survival (log-rank test on censored survival data $X^2_4 = 51.082$, $P < 0.0001$). Pretreatment by reduction of microbiota and treatment with probiotics alone did not have an effect, but survival was high for frogs that were not exposed to *Bd* and low for exposed frogs (Fig. 1a). Of the two treatments that were exposed to *Bd*, frogs that were not treated with probiotics survived a mean of 36.4 days, and those first treated with probiotics survived a mean of 31.2 days; survival was not significantly different between these two treatments (log-rank test on censored survival data $X^2_1 = 1.791$, $P = 0.181$).

Mass Change

A two-way ANOVA showed that *Bd*, but not probiotic treatment nor their interaction, had a significant effect on body mass ($F_3 = 8.163$, $P = 0.0001$). Frogs exposed to *Bd* tended to lose mass while unexposed frogs gained mass (Fig. 1b).

Culture-Independent Microbial Community Analysis

Microbial DNA from skin swabs taken 75 days after the last probiotic treatment (65 days after Bd exposure) was analyzed by DGGE. Levels of all four probiotic bacteria, *Pedobacter cryoconitis*, *Pseudomonas fluorescens*, *Chryseobacterium* sp., and *Iodobacter* sp., originally cultured from the study species in the wild [34], were below detectable limits with no bands matching the bacterial isolate standards, and no visually apparent differences in the microbial community profiles between probiotic-treated and control frogs. While the DGGE method lacks the power to detect small changes in community structure, no striking differences in the microbial community persisting longer than 2 months following biotherapy were detected.

Peptide Recovery and Bd Growth Inhibition

Peptides were recovered from two groups of ten frogs at the end of the experiment. Both groups had been pretreated to reduce microbes and were not exposed to *Bd*. One group (group 3) had probiotics added back, but the other did not (group 4). The quantity of peptides recovered did not differ significantly between probiotic-treated (mean ± SE = 1610 ± 141 μg/gbm) and control frogs (1754 ± 188 μg/gbm; independent *t* test, $t_{18} = -0.612$, $P = 0.548$; Fig. 1c). Peptides from probiotic-treated frogs inhibited *Bd* somewhat less than control frogs, although the difference in *Bd* growth inhibition was not significantly different between probiotic-treated frogs (mean % ± SE = 32.8 ± 9.7) compared to control frogs (51.9 ± 11.6; independent *t* test, $t_{18} = -1.268$, $P = 0.221$; Fig. 1d). Thus, skin peptide defenses against *Bd* were not enhanced by the microbial biotherapy regimen used here.

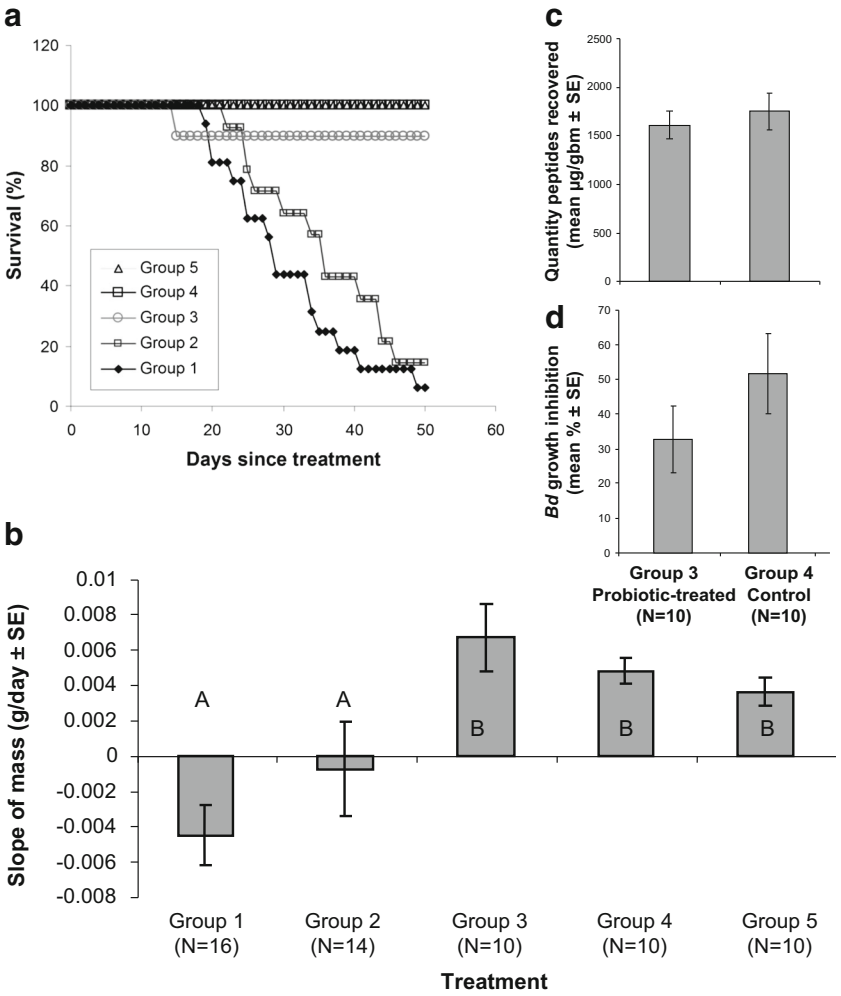

Fig. 1 Responses of juvenile Sierra Nevada yellow-legged frogs, *Rana sierrae*, to probiotic therapy. **a** Survival upon exposure to *Batrachochytrium dendrobatidis* (*Bd*) zoospores following each of five treatment regimens. Group descriptions can be found in Table 1. Frogs in groups 1 and 2 were exposed to *Bd* and experienced significant mortality. Probiotics were added to frogs in groups 1 and 3. Symbionts were reduced before the experiment in all cases except in group 5. **b** The change in mass following exposure to *Bd* in each of five treatment regimens. A 2 fixed-factor ANOVA showed that *Bd* had a significant effect on slope of mass change, but the factors probiotics and *Bd**probiotics interaction were not significant. Frogs exposed to both probiotics and *Bd* lost the most mass during the experiment. **c** Total quantity of skin peptides recovered from *R. sierrae* controls, or frogs treated with probiotic bacteria. **d** *Bd* growth inhibition caused by skin peptides collected from control and probiotic-treated *R. sierrae*

Comparison of Peptide Profiles Between Treatments

As shown in Fig. 2a and Table 2, the relative intensities of all but one peptide were similar between treatments. A peptide of mass 2524.5 Da was present at high intensities in control frogs, but almost absent in probiotic-treated frogs (Fig. 2a). The mean relative intensity of this peptide measured by MALDI-MS was 5.9% for group 3 probiotic-treated frogs and 41.3% for group 4 control frogs (Mann-Whitney $U = 81.0$, $N = 20$, $P = 0.019$). We confirmed the presence of the peptide in 7 of 19 samples by HPLC-MS (one sample was depleted); one of ten probiotic-treated frogs expressed this peptide, and six of ten control frogs expressed the peptide (Fisher's exact test, $P = 0.029$). Mean relative intensity of the peptide as measured by HPLC-MS was 12.0% in probiotic-treated frogs and 95.8% in control frogs (Mann-Whitney $U = 66.0$, $N = 19$, $P = 0.047$). Mean intensities of other peptides did not differ between treatments ($P > 0.05$, Table 2). Since samples were collected simultaneously by the same method, frogs appeared to downregulate this peptide (2524.5 Da, hereafter brevinin-1Ma) after exposure to the bacterial consortium. Relative intensities of peptides analyzed by MALDI-MS strongly correlated with the HPLC peak area (Pearson correlation $r = 0.971$, $P < 0.001$; Fig. 2b).

Identification of Downregulated Peptide

The 24-amino acid sequence of the peptide of mass 2524.5 (Supplementary Fig. S1) was determined by MALDI

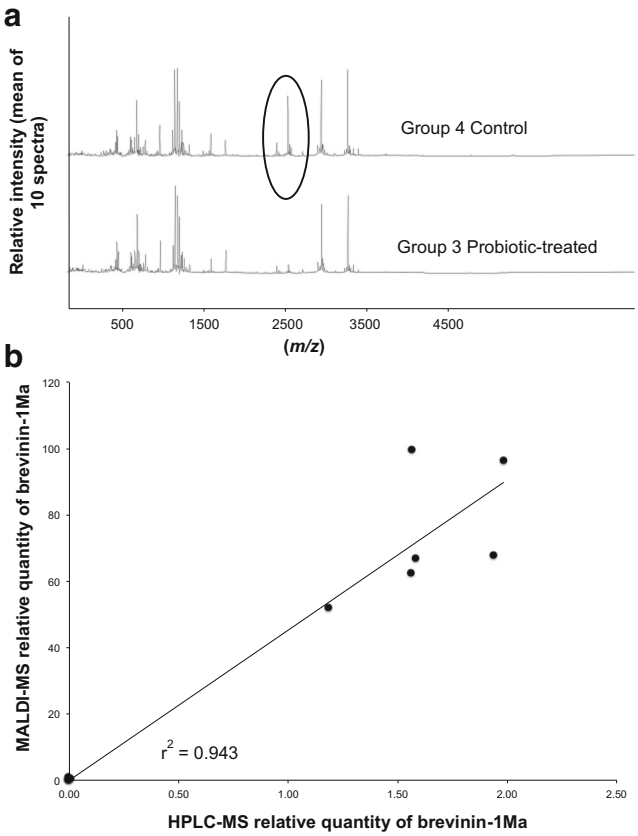

Fig. 2 Skin peptides from *Rana sierrae* examined by mass spectrometry. **a** MALDI mass spectra of skin peptides from probiotic-treated and control *R. sierrae* (groups 3 and 4 in Table 1) showing downregulation of one peptide, brevinin-1Ma, of approximate mass 2524.5 Da. **b** Relative intensity of brevinin-1Ma analyzed by MALDI-MS strongly correlates with HPLC peak area (Pearson correlation $r = 0.971$, $P < 0.001$)

tandem MS and Edman degradation to be FLPILAGLAANLVPKLICSITKKC (Supplementary Fig. S2). A disulfide bond creating a cysteine bridge is apparent from the structure. This peptide is in the brevinin-1 family, and is not an unexpected discovery based on findings from other species in the genus *Rana* [42]. The closest match in the antimicrobial peptide database [40] is 87.5% similarity to brevinin-1AUa (AP01436) from *R. aurora* (Fig. 3).

Inhibition of Bd and Bsal Growth by Brevinin-1Ma

The minimal inhibitory concentration (MIC), or concentration needed to completely inhibit growth of *Bd* zoospores, is between 6.25 and 12.5 μM for four previously tested brevinin-1 peptides described from other *Rana* species [43] (Table 3). Similarly, the MIC of brevinin-1Ma was within that range (12.5 μM) for two strains of *Bd*, and significant inhibition of *Bd* growth was observed at all concentrations tested, as low as 6.25 μM (Fig. 4). By contrast, a synthetic control peptide with a slightly altered structure (FLPIIAGIAANIVPKIICSITKKC) did not inhibit *Bd* at the tested concentrations up to 200 μM. Similarly, *Bsal* was also inhibited at both concentrations of brevinin-1Ma tested (Fig. 5).

Inhibition and Facilitation of Amphibian Skin Microbes by Brevinin-1Ma

One symbiotic fungus, *Penicillium* sp., cultured from amphibian skin was tested for effects on growth upon incubation with brevinin-1Ma; growth was not significantly different among doses (Fig. 5). Of the six probiotic bacterial cultures tested, growth of five of the bacteria was significantly affected by brevinin-1Ma (Fig. 5): *Chryseobacterium indologenes* was not significantly affected, whereas growth was facilitated for *C. ureilyticum*, *Serratia grimesii*, *Pseudomonas* sp., and *Janthinobacterium lividum*. Growth of *Rhodococcus fasciens* was significantly reduced upon incubation with brevinin-1Ma (Fig. 5).

Discussion

Here, we describe brevinin-1Ma, a novel skin defense peptide from the endangered Sierra Nevada yellow-legged frog, *R. sierrae*. The expression of the peptide was significantly affected by treatment with probiotics, such that

Table 2 Peptides from *Rana sierrae* skin quantified by HPLC-MS. Seven peptides, relative molecular mass (M_r), and retention time (RT) are indicated. Bold indicates significant difference between treatments (Mann-Whitney *U* test, $P = 0.047$)

Peptide	M_r	RT (min)	Mean area relative to bradykinin	
			Probiotic ($N = 10$)	Control ($N = 9$)
Bradykinin	1059.6	3.87	1	1
Temporin-1M	1367.9	10.15	3.15	3.59
Temporin-1M (free acid)	1368.9	9.96	4.59	3.44
Unknown	1698.7	5.73	0.10	0.13
Brevinin-1Ma	2524.5	11.7	**0.12**	**0.96**
Ranatuerin-2Mb	2928.6	8.36	0.63	0.56
Ranatuerin-2Ma	3272.9	8.24	0.84	0.92

	AP01436 Brevinin-1AUa, ***Rana aurora***
Alignment Result:	F L P I L A G L A A **K** L V P K **V F** C S I T K K C
Brevinin-1Ma:	F L P I L A G L A A **N** L V P K **L I** C S I T K K C
	Brevinin-1Ma, ***Rana sierrae***

Fig. 3 Alignment of brevinin-1Ma from *R. sierrae* to brevinin-1AUa from *R. aurora.* This is the closest match (87.5% similarity) out of 3053 entries in the antimicrobial peptide database [41]

addition of a consortium of four anti-*Bd* bacteria led to the apparent relative reduction of the peptide. The composition of the peptide profile, but not the quantity of total peptides, was altered by the therapy. In line with our hypothesis that microbial conditions influence expression of defense peptides, and that defense peptides help to regulate and maintain the host microbiota, we observed a shift in peptide profiles upon probiotic applications and the maintenance of skin microbial communities. A previous study of amphibian skin defense peptides showed that growth of the natural microbiota was facilitated, indicating a mechanism by which hosts may be selecting for certain skin microbiome composition or function [44]. Thus, we suggest that "antimicrobial peptide" is an outdated and misleading term. Instead, peptides such as brevinin-1Ma may have promicrobial functions and can facilitate growth of bacteria that are antagonistic toward *Bd* (Fig. 5). In addition, brevinin-1Ma has selective antibacterial and antifungal functions and can inhibit both *Bd* and *Bsal* directly, and this capacity is not broad spectrum but taxa-specific even among fungi, with *Penicillium* sp. unaffected.

A consortium of probiotic bacteria was tested for use as a prophylactic treatment against chytridiomycosis in *R. sierrae*. Although previous studies found a significant survival effect using skin applications of the Gram-negative bacterium *Janthinobacterium lividum* [25], we did not observe this result with a mixture of four phylogenetically distinct probiotic bacteria (two Bacteriodetes and two Proteobacteria), despite the potency of these bacteria at inhibiting *Bd* in co-culture experiments. Unlike the previous study, probiotics did not establish or were cleared while *Bd* proliferated within the skin. A reduction in the antifungal brevinin-1Ma peptide upon application of a relatively large dose of probiotic bacteria suggests that host immune defenses may have responded to this perceived bacterial threat. Changes in synthesis of skin defense peptides may have improved the host ability to inhibit bacteria, but did not increase inhibition of the fungal pathogen. Although the applied bacteria originated from wild adult *R. sierrae* and had anti-*Bd* properties in vitro [34], one or all of these bacteria may also present a health challenge to the host when applied at high concentrations. This challenge was effectively controlled by hosts that were not also infected with *Bd* (Fig. 1). Since brevinin-1Ma, like other brevinin peptides tested, has antifungal properties, disease resistance may have been improved if this peptide had increased, rather than decreased, on the skin.

Although the probiotic regimen tested did not benefit the host in terms of survival, mass, or antimicrobial skin peptide defense, several observations from this study may improve future research in this direction. First, pretreatment with antibacterial detergent and hydrogen peroxide, although not directly harmful (Fig. 1), may remove the protective mucosal barrier and thus force a more aggressive host immune response to any new invaders, even potentially beneficial probiotics. Alternatively, the frequent agitation of the host to assure exposure to the probiotic bacteria may have induced peptides that interfered with colonization. Second, a probiotic consortium was thought to increase the anti-*Bd* potential of the microbial community, and included *Pseudomonas* known to be relatively more abundant on uninfected *R. sierrae* in the field [45]. However, competition among the bacteria may have inhibited establishment. Thus, desirable properties of probiotic therapy include use of a persistent microbe that outcompetes others and defies resilience mechanisms of the host-microbial community such that a new community is permanently established, or an alternative stable state of the microbiome develops in which the probiotics may or may not be present, but the microbiome as a whole shifts toward greater anti-Bd function. Other probiotic regimens used in agriculture, aquaculture, and humans [46–51] apply repeated treatments to maintain the beneficial bacterial populations. Though effective for some captive amphibians as well [52], repeated treatments are less desirable for management of wild amphibians, where long-term

Table 3 Minimal inhibitory concentrations (MIC) of brevinin peptides, or the concentration needed to completely inhibit growth of *Batrachochytrium dendrobatidis* zoospores. In bold, data from this study

Species	Peptide	MIC (μM)*
Rana boylii	Brevinin-1BYa	12.5
R. boylii	Brevinin-1BYb	6.25
R. tarahumarae	Brevinin-1TRa	12.5
R. ornativentris	Brevinin-2Ob	6.25
R. sierrae	**Brevinin-1Ma**	**12.5**

*Data from species other than *R. sierrae* reviewed in Rollins-Smith and Conlon [43]

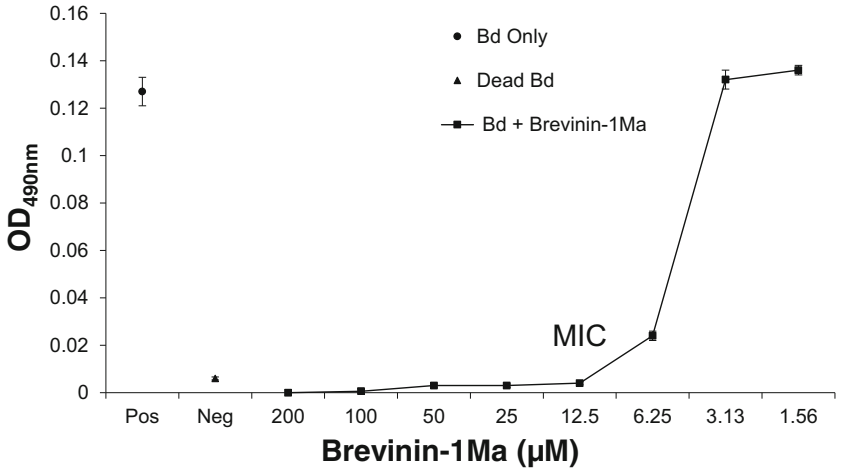

Fig. 4 Growth inhibition of the fungus *B. dendrobatidis* (*Bd*) caused by increasing concentrations of brevinin-1Ma (FLPILAGLAANLVPKLICSITKKC). Minimal inhibitory concentration (MIC) indicates the lowest concentration at which no *Bd* growth occurred. Similar curves were produced against *Bd* isolates JEL 197 and CJB 215. No *Bd* inhibition was observed when testing an artificial control peptide (FLPIIAGIAANIVPKIICSITKKC) replacing 4 leucines with isoleucines at the same concentrations

persistence in nature is needed to defend the host against future exposure to *Bd*. However, repeated treatments could be used in the laboratory or in captive breeding facilities, particularly when the aim is to treat infection rather than to prevent infection.

The use of bacteria that produce antifungal metabolites such as *J. lividum* [25, 41] may provide the best protection against *Bd*. Other mechanisms of host protection include direct competition with pathogens for space or resources, and indirect competition by inducing host immune defenses that are more effective against the pathogen than the probiont. (See Reid et al. [49] for descriptions of additional mechanisms leading to a balanced microbiota and restoration after insult.) For immune enhancement, we suggest experiments to explore the use of probiotic fungi, or a microbe that increases antifungal host defenses specifically. Several studies have found that the use of "nutrients," including vitamin D and sodium butyrate, can increase AMP expression [10, 11], and may assist with microbiome recovery from low-diversity dysbiosis [12]. Microbial

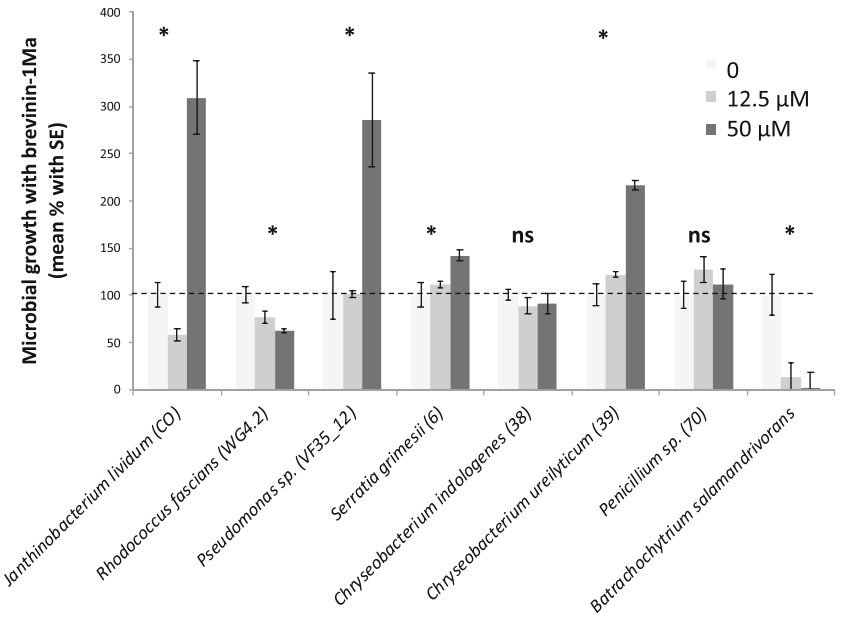

Fig. 5 Microbial growth measured with increasing concentrations of brevinin-1Ma. Six probiotic bacterial isolates from amphibian skin and two fungal isolates including the chytrid pathogen *Batrachochytrium salamandrivorans* (*Bsal*) were tested. Like *Bd* (Fig. 4), *Bsal* growth was inhibited by concentrations of at least 12.5 μM brevinin-1M. Here, probiotic is defined as capable of inhibiting *B. dendrobatidis* in co-culture assays. Dashed line indicates 100% growth with no addition of brevinin-1Ma. * indicates significant differences ($P < 0.05$) among concentrations tested by ANOVA

products that increase mucosal immunity are implemented in mucosal vaccines [53]. To induce antimicrobial peptides [2], we also suggest future amphibian disease studies focus on "prebiotics," or compounds that stimulate beneficial bacteria already present on the host to the disadvantage of pathogens. This has been used effectively against *Proprionibacteria acnes* on human skin, for example [54].

Finally, it is clear from this study that environmental conditions including microbial exposure are important for the relative rates of synthesis of amphibian defense peptides. Thus, researchers focused on discovery of antimicrobial skin peptides from amphibians may consider collecting peptides under several host and environmental conditions. One risk or opportunity of probiotic therapy identified here is an unexpected interaction with host immunity. With methodological improvements, altering the microbial environment as modeled here on amphibian skin may be a simple method to induce host antimicrobial peptides and enhance immune defense.

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Affiliations

Douglas C. Woodhams[1] · Louise A. Rollins-Smith[2,3] · Laura K. Reinert[2] · Briana A. Lam[4] · Reid N. Harris[4] · Cheryl J. Briggs[5] · Vance T. Vredenburg[6] · Bhumi T. Patel[1] · Richard M. Caprioli[7] · Pierre Chaurand[8] · Peter Hunziker[9] · Laurent Bigler[10]

1 Department of Biology, University of Massachusetts Boston, Boston, MA 02125, USA

2 Departments of Pathology, Microbiology and Immunology and Pediatrics, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

3 Department of Biological Science, Vanderbilt University School of Medicine, Nashville, TN 37235, USA

4 Department of Biology, James Madison University, Harrisonburg, VA 22807, USA

5 Department of Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, CA 93106-9610, USA

6 Department of Biology, San Francisco State University, San Francisco, CA 94132-1722, USA

7 Mass Spectrometry Research Center and Department of Biochemistry, Vanderbilt University, Nashville, TN 37232-8575, USA

8 Department of Chemistry, Université de Montréal, Montreal, QC H3T 1J4, Canada

9 Functional Genomics Center Zurich, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

10 Department of Chemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland