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Interacting Symbionts and Immunity in the Amphibian Skin Mucosome Predict Disease Risk and Probiotic Effectiveness

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Abstract
Pathogenesis is strongly dependent on microbial context, but development of probiotic therapies has neglected the impact of ecological interactions. Dynamics among microbial communities, host immune responses, and environmental conditions may alter the effect of probiotics in human and veterinary medicine, agriculture and aquaculture, and the proposed treatment of emerging wildlife and zoonotic diseases such as those occurring on amphibians or vectored by mosquitoes. Here we use a holistic measure of amphibian mucosal defenses to test the effects of probiotic treatments and to assess disease risk under different ecological contexts. We developed a non-invasive assay for antifungal function of the skin mucosal ecosystem (mucosome function) integrating host immune factors and the microbial community as an alternative to pathogen exposure experiments. From approximately 8500 amphibians sampled across Europe, we compared field infection prevalence with mucosome function against the emerging fungal pathogen Batrachochytrium dendrobatidis. Four species were tested with laboratory exposure experiments, and a highly susceptible species, Alytes obstetricans, was treated with a variety of temperature and microbial conditions to test the effects of probiotic therapies and environmental conditions on mucosome function. We found that antifungal function of the amphibian skin mucosome predicts the prevalence of infection with the fungal pathogen in natural populations, and is linked to survival in laboratory exposure experiments. When altered by probiotic therapy, the mucosome increased antifungal capacity, while previous exposure to the pathogen was suppressive. In culture, antifungal properties of probiotics depended strongly on immunological and environmental context including temperature, competition, and pathogen presence. Functional changes in microbiota with shifts in temperature provide an alternative mechanistic explanation for patterns of disease susceptibility related to climate beyond direct impact on host or pathogen. This non-lethal management tool can be used to optimize and quickly assess the relative benefits of probiotic therapies under different climatic, microbial, or host conditions.


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Introduction
Probiotic therapies often aim to extend or shape the immune function of hosts by altering the symbiotic microbial community. Probiotics are used in human and veterinary medicine, agriculture and aquaculture, and have been proposed for treatment of emerging wildlife diseases such as those occurring on corals and amphibians [1,2]. Microbiota can mediate pathogenesis through a range of mechanisms [3,4], and disease ecology studies demonstrate that parasitic and non-parasitic microbes interact with each other and with the host immune system such that pathogenicity is often influenced by environmental conditions [5–8]. Thus, the environment affects the risk of disease to individuals, populations, and species, and assessing disease risk under changing conditions is vital to conservation and infectious disease mitigation and can direct the allocation of resources for most effect [9–12].
The microbiota inhabiting skin and mucosal surfaces has a profound impact on host health and immunity [7,13,14], and may be predictive of risk for some diseases [15–17]. Amphibian skin is a model system for diseases affecting vertebrate mucosa. The mucosae, or micro-ecosystem of the mucus, as defined here contains interdependent host factors (mucosal antibodies, antimicrobial peptides, lysozyme, alkaloids) and microbial-community factors (microbiota, antibiotic metabolites). The mucosae has various functions potentially including communication, and predator and pathogen defense. Here, we develop a non-lethal assay and holistic measure referred to as "mucosome function" to describe the effect of amphibian skin mucus on pathogen viability. We examine how environmental and immunological contexts may impact the outcome of host-microbe symbioses, and how mucosome function captures the in vivo complexity of the micro-ecosystem and can thus accurately predict susceptibility to infection. We focus on probiotic bacteria and fungi applied to the skin mucosae as biocontrol agents against the emerging amphibian disease chytridiomycosis.

Chytridiomycosis is a major cause of global amphibian population declines and species extinctions [18,19]. The disease is caused by the chytrid fungus Batrachochytrium dendrobatidis, or Bd, and is strongly influenced by climatic conditions [20]. Climate-linked changes to the entire microbiota, not just Bd, may influence disease susceptibility [5]. Current efforts to mitigate chytridiomycosis in wildlife populations have turned to bioaugmentation, or the use of probiotic therapies [1,21]. The successful prophylactic infection and community and immunological interactions. Because it can establish and persist on the host in its natural environmental context is urgently needed.

Our aims in this study were (1) to develop a holistic, simple, non-invasive, and non-lethal method to measure mucosome function against Bd. Using this tool, we aimed (2) to test whether mucosome function can predict Bd infection prevalence of amphibians in the field and survival in Bd exposure experiments. While we show that probiotics are influenced by a variety of factors including competition, temperature, and innate immunity when tested in vitro, we aimed (3) to use mucosome function as an ecologically-integrated predictor of probiotic therapy effect so that future research can test probiotic strategies for conservation and not lose hope in the potential of probiotic therapy in the face of immunological and ecological complexity. We provide a detailed protocol for measuring mucosome function in File S1.

Materials and Methods

Ethics statement

Permits to conduct fieldwork were obtained from the Swiss cantonal conservation authorities, and from Germany - German federal licence (Rheinland-Pfalz) no. 425-104.143.0904 Struktur- und Genehmigungsdirektion Nord, Koblenz. All animal procedures were approved by the Veterinary Authority of Zurich (110/2007 and 227/2007) and the Federal Office for the Environment. Fieldwork conformed to standard decontamination practices to avoid transport of pathogens between sites. All animals in experiments were monitored daily for animal welfare and to ameliorate suffering. During experiments, any individual demonstrating clinical signs of disease including lethargy, abnormal skin shedding, and loss of righting reflex were humanely euthanized. At the end of the experiment, all animals were humanely euthanized by overdose of tricaine methanesulfonate.

Survey of Bd infection prevalence

To compare Bd infection prevalence among species and life-history stages, we combine previously unpublished results from field studies in Switzerland with Bd surveys from amphibians across Europe collated by Bd-Maps (www.bd-maps.net, accessed September 1, 2013). In addition to data from 5939 sampled amphibians available from Bd-maps, skin swabs were collected from 2591 amphibians from 12 species and from 66 Bd-positive populations from the northern parts of Switzerland and tested for Bd between 2007 and 2009 (Table 1). Amphibians were caught by risk and treatment effects in rare amphibians including relict populations or captive populations of endangered species intended for reintroduction.

Typical approaches to compare species susceptibility and to assess disease risk include pathogen exposure experiments [27], or field surveys to compare infection prevalence and monitor disease and population trajectories [20], or modeling environmental and biogeographic risk factors [10,29]. Deficits of conventional pathogen exposure experiments include lack of environmental context when amphibians are exposed under clean laboratory conditions. Biodiversity including microbiota and macrobiota can influence disease outcome [30], and bacterial community diversity is reduced through time in captivity without natural sources such as soil for re-inoculating the skin [31]. The exposure history, population genetics, and life-history stage of the amphibians used in the experiment, as well as the strain and dose of the pathogen can all affect experimental outcomes, and many threatened species are not suitable for such experiments. In addition, growth of Bd is often inhibited by skin microbiota of amphibians [32,33]. However, little is known about how protective microbiota differs among host populations or regions, or how mucosome function is altered by enrichment with potential probiotics.
dip-netting and swabbed with a sterile cotton swab (Copan Italia S.p.A., Brescia, Italy). Field material was cleaned and disinfected before moving between different sites to avoid contamination and spread of Bd and other pathogens. Extraction and analysis for Bd-DNA were done following the qPCR protocol by Boyle et al. [34] using Bd-specific primers and standards to quantify the amount of DNA. We ran each sample twice and the PCR was repeated if the two wells returned dissimilar results. Reactions below 1 genomic equivalent were scored Bd-negative to avoid false positives. Mean infection prevalence with 95% binomial confidence interval was calculated for each species and life stage sampled, and calculated for both Europe and Switzerland.

**Bd infection prevalence predicted by skin defenses**

Skin defense peptides and mucosome samples were tested against Bd for comparison of anti-Bd activity with infection prevalence in natural populations by logistic regression in R. Amphibians sampled for skin peptides and mucosome function (Table 1) were sampled in Switzerland and compared to field infection prevalence from Switzerland and across Europe in separate analyses. Skin peptides were collected upon induction by subcutaneous injection of metamorphosed amphibians with 40 nmole/g body mass norepinephrine (bitartrate salt, Sigma) or immersion of larval amphibians in 100 μM norepinephrine, and tested for Bd growth inhibition as previously described [35,36]. Skin peptide samples from post-metamorphic amphibians only were used in the logistic regression analyses because different methods of peptide induction were used on larval stages. Mucosome samples from multiple life-history stages of the same species were included and matched to life-history stages sampled for Bd diagnostics (Table 1). Detailed methods for measuring mucosome function against Bd using a fluorescence assay of Bd viability adapted from Stockwell et al. [37] (Fig. S1 in File S1) and comparisons of mucosome function and skin peptide defenses against Bd are presented in Supporting Information (Figs. S4, S5 in File S1).

**Survival predicted by mucosome function**

To examine the relationship between mucosome function against Bd and susceptibility to infection and subsequent survival we performed experimental exposures to Bd on four species. All animals were exposed to zoospores from Swiss lineage Bd TG 739 isolated from a moribund *A. obstetricans* in Gamlikon, Switzerland in 2007 [38] and cryopreserved until use. Egg clutches were obtained from *A. obstetricans* (n = 45), and *B. variegata* (n = 8), *A. obstetricans* isolated from a moribund animal and *B. variegata* for the environmental context experiments described in 2007 [38] and cryopreserved until use. Egg clutches were obtained from *A. obstetricans* (n = 45), and *B. variegata* (n = 8), *A. obstetricans* isolated from a moribund animal and *B. variegata* for the environmental context experiments described in 2007 [38] and cryopreserved until use. Egg clutches were obtained from *A. obstetricans* (n = 45), and *B. variegata* (n = 8), *A. obstetricans* isolated from a moribund animal and *B. variegata* for the environmental context experiments described in 2007 [38] and cryopreserved until use. Egg clutches were obtained from *A. obstetricans* (n = 45), and *B. variegata* (n = 8), *A. obstetricans* isolated from a moribund animal and *B. variegata* for the environmental context experiments described in 2007 [38] and cryopreserved until use. Egg clutches were obtained from *A. obstetricans* (n = 45), and *B. variegata* (n = 8), *A. obstetricans* isolated from a moribund animal and *B. variegata* for the environmental context experiments described in 2007 [38] and cryopreserved until use. Egg clutches were obtained from *A. obstetricans* (n = 45), and *B. variegata* (n = 8), *A. obstetricans* isolated from a moribund animal and *B. variegata* for the environmental context experiments described in 2007 [38] and cryopreserved until use. Egg clutches were obtained from *A. obstetricans* (n = 45), and *B. variegata* (n = 8), *A. obstetricans* isolated from a moribund animal and *B. variegata* for the environmental context experiments described in 2007 [38] and cryopreserved until use. Egg clutches were obtained from *A. obstetricans* (n = 45), and *B. variegata* (n = 8), *A. obstetricans* isolated from a moribund animal and *B. variegata* for the environmental context experiments described in 2007 [38] and cryopreserved until use.

Toadlets were of similar size (mean ± SD: 2.1 ± 0.3 g; ANOVA, F2 = 1.179, P = 0.332) and treated at the same time with one exception. Toadlets in the Bd-exposure group were exposed to Bd approximately 2 months prior to the microbial exposure treatments, and the toadlets were smaller (1.5 ± 0.3 g), and no longer infected at the time of sampling based on qPCR.

We treated recently metamorphosed common midwife toads, *Alytes obstetricans* (N = 70; 10 per treatment group, 7 treatments), by housing them individually at 5°C, 18°C, or 25°C with no microbes added, or at 18°C with exposure to Bd zoospores (8.5×10^6 zoospores of global panzootic lineage isolated from a Bufo bufo in the UK [38]), a probiotic fungus *Penicillium expansum*, or a probiotic bacterium *P. fluorescens* or *F. johnsoniae*. Toadlets were bathed individually for one hour in water containing the microbes and after 2 weeks, toadlets from all treatments were sampled on the same day for mucosome function and subsequently skin peptides, sampled as described above.

**Temperature, competition of probiotic strains, and co-culture with Bd**

To determine the effects of competitive interactions and temperature on probiotic potential, 11 common host-associated isolates were chosen. These included two isolates of *Serratia plymuthica* and one isolate of *Janthinobacterium lividum* from egg clutches of midwife toads, three isolates of *Flavobacterium johnsoniae* and five species of *Pseudomonas* isolated from the skin of adults. Based on 16S rRNA gene sequences, all 11 isolates were considered unique operational taxonomic units (OTUs) at 99%, but clustered into 7 OTUs at 97% similarity as determined by the UCLUST algorithm in QIIME. The 16S rRNA gene sequences of all isolates were deposited in the European Nucleotide Archive (Table S1 in File S1).

In one set of experiments, bacterial isolates were freshly grown at 18°C on RIIA agar media supplemented with 1% tryptone then transferred to experimental conditions. Bacteria and Bd (Swiss isolate TG 739) readily grew on the same media. Plate experiments were performed in duplicate. Both isolates of *Serratia plymuthica* were grown separately at 18 and 25°C, or at 18°C on media inoculated with Bd and allowed to dry before streaking the bacteria. Two isolates of *F. johnsoniae* were grown separately or combined on media inoculated with Bd, and grown at 18°C. When combined, each isolate was streaked across the entire plate. Three *Pseudomonas* isolates were grown either separately, combined, or combined on media inoculated with Bd, and grown at 18°C. Control plates of sterile media or Bd-only were also tested. All plates were incubated for 3 days, and then rinsed with 2 ml sterile
<table>
<thead>
<tr>
<th>Species</th>
<th>Life-history stage#</th>
<th>Peptide effectiveness* (N)</th>
<th>SE</th>
<th>Mean mucosome function against Swiss Bd (N)</th>
<th>SE</th>
<th>Switzerland: Percent infected (N)</th>
<th>95% binomial confidence interval</th>
<th>Europe: Percent infected (N)</th>
<th>95% binomial confidence interval</th>
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</thead>
<tbody>
<tr>
<td>Alytes obstetricans</td>
<td>Adult/Subadult</td>
<td>15.92 (8)</td>
<td>6.21</td>
<td>0.012 (10)</td>
<td>0.00</td>
<td>4.9 (41)</td>
<td>0.6–16.5</td>
<td>29.7 (209)§</td>
<td>23.5–36.4</td>
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<td>Alytes obstetricans</td>
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<td>37.75 (9)</td>
<td>12.15</td>
<td></td>
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<td></td>
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<tr>
<td>Alytes obstetricans</td>
<td>Larvae</td>
<td>48.76 (5)</td>
<td>24.23</td>
<td>2.963 (10)</td>
<td>0.681</td>
<td>45.4 (2111)</td>
<td>43.3–47.6</td>
<td>38.0 (3008)</td>
<td>36.3–39.8</td>
</tr>
<tr>
<td>Bombina variegata</td>
<td>Adult/Subadult</td>
<td>1.075 (4)</td>
<td>0.081</td>
<td>2.00 (150)</td>
<td>0.081</td>
<td>13.9–27.3</td>
<td>21.1 (227)</td>
<td>16.0–27.0</td>
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<tr>
<td>Bufo bufo</td>
<td>Adult</td>
<td>16.34 (15)</td>
<td>3.7753</td>
<td>0.117 (9)</td>
<td>0.082</td>
<td>0.0 (22)</td>
<td>0.0–15.4</td>
<td>0.9 (3606)</td>
<td>0.6–1.2</td>
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<td>Bufo bufo</td>
<td>Larvae</td>
<td>1.284 (5)</td>
<td>0.404</td>
<td></td>
<td></td>
<td>6.7 (45)</td>
<td>1.4–18.3</td>
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<tr>
<td>Hyla arborea</td>
<td>Adult</td>
<td>11.42 (7)</td>
<td>2.15210</td>
<td></td>
<td></td>
<td>3.8 (26)</td>
<td>0.1–19.6</td>
<td>12.5 (32)</td>
<td>3.5–29.0</td>
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<tr>
<td>Ichthyosaura alpestris</td>
<td>Adult</td>
<td>0.94 (7)</td>
<td>0.52546</td>
<td>1.361 (20)</td>
<td>0.062</td>
<td>24.8 (629)</td>
<td>21.5–28.4</td>
<td>21.5 (775)</td>
<td>18.7–24.6</td>
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<td>Lissotriton vulgaris</td>
<td>Adult</td>
<td>1.85 (4)</td>
<td>1.02506</td>
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<td></td>
<td>27.3 (22)</td>
<td>10.7–50.2</td>
<td>17.0 (47)</td>
<td>7.7–30.8</td>
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<td>27.27 (10)</td>
<td>3.18</td>
<td></td>
<td></td>
<td>22.4 (170)</td>
<td>16.3–29.4</td>
<td>15.6 (275)</td>
<td>11.6–20.5</td>
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<td>0.042</td>
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<td>6.1–23.3</td>
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<td>1.97 (13)</td>
<td>0.62111</td>
<td>0.251 (10)</td>
<td>0.128</td>
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<td>0.120</td>
<td></td>
<td></td>
<td>0.0 (20)</td>
<td>0.0–16.8</td>
<td>0.0 (23)</td>
<td>0.0–14.8</td>
</tr>
<tr>
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<td>1.32654</td>
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<td></td>
<td>11.1 (9)</td>
<td>0.3–48.3</td>
<td></td>
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<tr>
<td>Salamandra salamandra</td>
<td>Larvae</td>
<td>42.78 (5)</td>
<td>13.35528</td>
<td></td>
<td></td>
<td>23.2 (69)</td>
<td>13.9–34.9</td>
<td></td>
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</tbody>
</table>

Skin peptide effectiveness is the percent inhibition of Bd zoospore growth caused by 50 µg/ml peptide multiplied by the quantity of peptides (mg) per g amphibian according to Woodhams et al. [11]. The mucosome function against Bd (Swiss isolate TG 739) is a measure of zoospore viability quantified by the ratio of green:red fluorescence as described above. Infection prevalence is the mean from all amphibians in each group from multiple sites and seasons.

*Peptide effectiveness = % inhibition of Bd growth at 50 µg/ml * mg peptides/g frog mass.

Includes samples from chytridiomycosis outbreak sites in Spain (S. Walker, unpubl.), not included in logistic regression.

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media, and 6 negative control wells contained heat-killed *Bd* and control bacterial growth were tested by t-tests using a density measured at 480 nm. Differences between experimental water. Growth after 48 hr was measured as change in optical

Effects of host skin peptides and *Bd* metabolites on probiotics in culture

To test for the response of bacterial growth upon culture with either *Bd* filtrate or host skin peptides, bacteria were grown in RIIA liquid media on 96 well plates. Supernatant from a 2-week old culture of *Bd* (type isolate JEL [197] growing in 0.5% tryptone was filtered through a 0.22 μm syringe filter. An equal volume of *Bd* filtrate or sterile media was added to bacterial cultures. To test effects of peptides, we added an equal volume of sterile water or natural mixtures of partially-purified skin peptides from *A. obstetricans* (Table S2 in File S1). Percent inhibition depended on filtrate dose (see Results) and was not considered comparable among bacterial isolates.

Results

Survey of *Bd* infection prevalence

Surveys of approximately 8500 amphibians (http://www.bd-maps.net/; this study) at different life-history stages for *Bd* infection based on qPCR indicated high prevalence in larval midwife toads, *A. obstetricans* (45% infected in Switzerland) and aquatic adult newts *Ichthyosaura alpestris* (26%), and *Lissotriton vulgaris* (27%). Low infection prevalence (<5%) was detected in populations of adult *A. obstetricans*, *Bagor bufo*, *Rana temporaria*, and *Hyla arborea* (Table 1, Fig. 1).

*Bd* infection prevalence predicted by skin defenses

We examined two non-lethal measures of susceptibility to infection in pathogen-free Swiss amphibians acclimated to laboratory conditions. These included testing *Bd* growth or viability upon exposure to natural mixtures of partially purified skin defense peptides, and a holistic functional measure of the skin mucosal ecosystem (*mucosome function*) including ambient skin defenses: peptides, alkaloids, lysozymes, mucosal antibodies, microbiota and microbial metabolites [41]. Both antifungal skin peptides and mucosome function were correlated with infection prevalence in natural populations across Europe (Fig. 1a,c) and within Switzerland (Fig. 1b,d). Prevalence of infection with *Bd* decreased with peptide efficiency (Fig. 1c,d, logistic regressions: Europe, *P* = 0.0015; Switzerland, *P* = 0.0079). While induced peptide defenses stored in granular glands were measured here, ambient peptides (not induced by norepinephrine) are a natural component of the mucosome [42,43]. Mucosome function was tightly correlated to *Bd* prevalence in natural populations of Swiss amphibians (Fig. 1b, *P* = 0.0001) and in amphibians across Europe (Fig. 1a, *P* = 0.0020). The odds ratios of *Bd* colonization in Swiss amphibians was 1.950 (Europe, 2.969) with each unit change in mucosome function, and 0.839 (Europe, 0.811) with each unit decrease in skin peptide efficiency. Correlations of mucosome function and induced skin peptide efficiency are presented in Figure S4 in File S1 and suggest that both host and microbial factors contribute to mucosome function against *Bd*.

Survival predicted by mucosome function

Pathogen exposure experiments were conducted on four host species with a Swiss isolate of *Bd*, and relative survival post-metamorphosis of infected tadpoles differed among species (% relative survival, mean±SD days survived: *A. obstetricans* (0%, 24±17.5 d), *Bombina variegata* (39.0%, 32±22.9 d), and *Polypedilum esculentus* (30.4%, 12±12.8 d; Fig S2 in File S1). Relative survival of recently metamorphosed *Rana temporaria* exposed to *Bd* was 100% (Fig S3 in File S1), and no colonization by *Bd* was detected by qPCR (n = 92). Success of *Bd* colonization of tadpoles also differed among species (Pearson *χ*² = 13.102, *P* = 0.004): *A. obstetricans* (13.9% infected, n = 36), *B. variegata* (10.7%, n = 75), and *P. esculentus* (7.9%, n = 76). Mucosome function predicted survival (logistic regression, *P* = 0.0001; Fig 2a) and infection with *Bd* in these species (P = 0.0106; Fig 2b). The odds of infection increased by 1.751 with each unit change in mucosome function, and the odds of survival decreased by 0.0454.

Host ecological context and skin defenses

Midwife toads, *A. obstetricans*, were treated with various temperature and probiotic therapies and tested for mucosome function. Host context significantly affected mucosome permissiveness or lethality towards *Bd* (Fig 3a; ANOVA, *F*ₙ = 41.606, *P* < 0.001). *Bd* viability was similar following incubation with mucosome samples from toads at temperatures ranging from 5–25°C. Mucosome samples from toads previously exposed to *Bd* were less effective at killing *Bd* zoospores, while those from toads treated with probiotics *Flavobacterium johnsoniae* and *Penicillium expansum* were most effective at killing zoospores (Fig 3a). While *Pseudomonas* in general, and the *P. fluorescens* isolate (76.5c) used in this study were often effective at inhibiting *Bd* in co-culture and produced antifungal metabolites across a range of temperatures ideal for *Bd* growth (Fig 4a, Table S2 in File S1), there was no significant benefit of this probiotic when applied on hosts in terms of increasing mucosome function and reducing *Bd* viability (Fig 3a).

Because one significant antimicrobial component of *A. obstetricans* skin mucus is antimicrobial peptides (AMPs) [44], we collected peptide skin secretions, quantified them per surface area of the toads and measured their ability to inhibit *Bd* growth at a standardized concentration of 100 μg/ml. On average, toads produced 0.25 mg peptide per cm² surface area, and at 100 μg/ml these peptides inhibited *Bd* growth by 48.7%. These values did not differ significantly among treatment groups, nor did a combined measure of skin peptide effectiveness against *Bd* (% *mg/cm²*, Fig 3b; Kruskal-Wallis tests, *P* > 0.05). Thus, skin peptides stored in granular glands were not significantly affected by the 2-week temperature and microbe treatments including previous exposure.
to *Bd*. There was not a significant correlation between peptide effectiveness and mucosome function against *Bd* (Fig. S5 in File S1; Pearson, $\chi^2 = -0.102, P = 0.827$). Zoospore viability after exposure to mucosome samples was significantly higher in the *Bd*-exposure treatment compared to other treatments (Fig. 3a). However, skin peptides induced from hosts in the *Bd*-exposure treatment were effective at inhibiting *Bd* growth, and not significantly different than peptides from toads in other treatments (Fig. 3b).

**Temperature, competition of probiotic strains, and co-culture with *Bd***

Environmental conditions affected the capacity of probiotic bacteria to inhibit the fungal pathogen *Bd* (Table S2 in File S1). Two *Serratia plymuthica* isolates (isolates 27 and 28) were capable of inhibiting *Bd* growth when incubated at 18°C. Isolate 27 was inhibitory under all tested conditions: 18°C, 25°C, and 18°C co-cultured with *Bd*. Isolate 28 significantly enhanced *Bd* growth at 25°C, and was neither enhancing nor inhibitory at 18°C when co-cultured with *Bd* (Fig. 4c, Table S2 in File S1). A dose-response of *Bd* growth inhibition was found such that filtrate diluted 1/10 was...
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Effects of host skin peptides and Bd metabolites on probiotics in culture

Amphibian skin defense peptides may regulate the skin microbiota. We found that natural mixtures of skin peptides from A. obstetricans at a concentration of 100 μg/ml significantly inhibited growth of Pseudomonas migulae (73b1) and significantly enhanced growth of P. filiscindens (73c1), Flavobacterium johnsoniae (70d1), and Janthinobacterium lividum (73c1), while significantly enhancing the growth of J. lividum (77.5b) caused by bacterial filtrate was significantly different from control bacterial growth with water only added (independent t-tests, P < 0.001). In several cases, the proportion of infected frogs (95% binomial CI; b) predicted by Mucosome function (mean, SE) indicates significantly less inhibitory than undiluted filtrate (paired t-test, t_{15} = 9.836, P < 0.001), and filtrate from control plates with or without Bd significantly enhanced Bd growth (Table S2 in File S1). Testing metabolites of the bacteria growing at 14, 19, and 22°C in liquid culture against the global panzootic lineage of Bd showed similar results including a dose-response (Fig. 4a,b), paired t-test, t_{15} = -10.607, P < 0.001). In several cases, Bd growth was enhanced with addition of diluted bacterial metabolites in comparison to positive control growth with RIIA media only (>100%, Fig. 4b). Most cultures were more inhibitory of Bd at the lower temperatures, except for J. lividum, (isolate 77.5b) which was most inhibitory at 22°C (Fig. 4a,b).

While all bacteria were unique based on 16S rRNA gene sequencing when clustered at 99% similarity, probiotic physiology and function against Bd did not always correspond to OTU clustering at 97% similarity (Table S1 in File S1). In other words, bacterial isolates considered to be the same “species” based on 16S rRNA could have different antifungal function. Here, only one of two Flavobacterium johnsoniae isolates inhibited Bd growth. When grown together, the filtrate remained inhibitory. However, when grown together and co-cultured with Bd, the filtrate was no longer inhibitory. Three Pseudomonas isolates were capable of inhibiting Bd growth, and were inhibitory when combined with or without co-culture with Bd. The above mentioned growth inhibition of Bd caused by bacterial filtrate was significantly different from control bacterial growth with water only added (independent t-tests, P < 0.05 and replicated result; all data shown in Table S2 in File S1). These conditions represent infected or uninfected hosts and are illustrative rather than comprehensive of all possible environmental conditions and competitive interactions.

Discussion

We found that a holistic measure of mucosome function against Bd is predictive of infection risk in natural populations of amphibians and survival in laboratory exposure experiments. While induced antimicrobial peptides may explain some variation in infection risk (Fig. 1b,d), mucosome function can be altered through probiotic therapy (Fig. 3a), and thus microbial communities play a major role in determining susceptibility to infection with Bd. In particular, tadpoles of the endangered midwife toad, A. obstetricans may be most at risk of both infection and subsequent disease-induced mortality upon metamorphosis (Fig. 2), even though adult toads are well protected by the mucosome and perhaps resistant to colonization with Bd. Similarly, the common frog R. temporaria has strong mucosome activity against Bd, shows Bd colonization resistance, but has relatively poor skin defense peptides. This suggests that this common species has protective microbial communities. Adaptive defenses are not suspected because frogs were raised from eggs and had no history of exposure to Bd.

In this study, we provide several striking examples showing that probiotic capacity depends on immunological and environmental context. These examples lead to recommendations for choosing probiotics based on predictable host conditions. Temperature is known to influence amphibian host immune function [41] and
bacterial growth, metabolism, pigment and antibiotic production [45]. However, it was surprising that a shift from 18 to 25 °C, a typical natural range for midwife toads, caused a common bacterial symbiont of the eggs and skin, *Serratia plymuthica*, to change from inhibiting *Bd* to enhancing *Bd* growth (Fig. 4c).

Testing metabolites of the bacteria growing at 14, 19, and 22 °C in liquid culture against the global panzootic lineage of *Bd* showed similar results (Fig. 4b). Functional changes in probiotic activity with shifts in temperature have not previously been reported. Our results provide an alternative mechanistic explanation for patterns of susceptibility related to climate, which have previously been limited to empirical observation and pathogen-centered effects [46–49].

The microbial interactions we tested also altered antifungal effects relative to what would be predicted from individual isolates. For example, co-culture of *Flavobacterium johnsoniae* with *Bd* caused cultures of the bacterium that normally produce antifungal metabolites to switch off antifungal activity: when grown together with *Bd*, *F. johnsoniae* filtrate was benign, and indeed *Bd* filtrate inhibited the growth of two out of three *F. johnsoniae* isolates (Fig. 4c).
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**Figure a:** Growth of Bd upon exposure to bacterial metabolites (mean % of control with SS).

**Figure b:** Growth of Bd upon exposure to bacterial metabolites (mean % of control with SS).

**Isolate 27:**
- Supernatant effect on Bd growth: inhibitory
- **18°C:**
- **25°C:**

**Isolate 28:**
- Supernatant effect on Bd growth: inhibitory, enhancing
Figure 4. Environmental context determines antifungal capacity of probiotics. Tested temperatures (14, 19, 22 °C) significantly affected the production of bacterial metabolites in liquid media that could inhibit *B. dendrobatidis* (*Bd*; GPL isolate VMV 813) zoospore growth in a dose-dependent fashion (α = full strength metabolites, b = 1:10 dilution). * indicates that *Bd* growth differed among metabolite temperature treatments (ANOVA, Bonferroni-corrected P’s < 0.05). (c) Representative replicates are shown of two isolates of *Serratia plymuthica* isolated from egg clutches of common midwife toads, *Alytes obstetricans*, grown on solid media under different temperature conditions. Filtrate from isolate 27 always inhibited growth of *Bd*, but filtrate from isolate 28 inhibited *Bd* growth at 18 °C, and enhanced *Bd* growth at 25 °C. Filtrate from sterile media (R2A agar supplemented with 1% tryptone) caused enhanced growth of *Bd*. Note that colony color can be an indication of antifungal metabolites such as prodigines from red *Serratia* spp. [45,67], but are produced only under certain growth conditions. doi:10.1371/journal.pone.0096375.g004

S6 in File S1). Co-evolution of *Bd* and amphibian hosts is a postulated driver of pathogenicity factors including compounds suppressing host immune defenses [43,50,51]. These factors may extend to inhibiting certain antifungal symbionts or altering their function.

Myriad microbial and immune interactions occur once probiotics are added to living hosts. Thus, testing probiotics in *vivo* is critical for testing the intended antifungal effect of probiotic therapy under realistic environmental conditions. We found that previous exposure to *Bd* may have a negative effect on host immunity or the ability of the mucosome to kill zoospores (Fig 3A). This result is consistent with a study on Australia green-eyed tree frogs, *Litoria serrata*, showing inhibition of ambient skin peptides with *Bd* infection but no inhibition of inducible stored skin peptides [43]. Because stored skin defense peptides can have potent activity against *Bd*, yet not be active on the skin, induced skin peptides may not accurately predict infection susceptibility. This mystery of how seemingly well-defended species can be affected by chytridiomycosis [52] deserves careful study on the conditions under which host skin defense peptides are activated. Induced skin defense peptides were previously used to predict disease susceptibility in Panama [11] and New Zealand [53]. In Panama, most species had weak peptide defenses and declined after disease emergence while only two species had strong peptide defenses against *Bd* compared to reference species of known disease resistance. Of these two species, the one with the highest levels of skin peptide defenses persisted at the field site (*Espadarana prosoblepon* [54], and the other species (*Agalychnis lemur*) disappeared, but a relict population has been detected nearby (Julie Ray, pers. comm.). In New Zealand, all native species demonstrated high levels of skin peptide defenses and appear to resist chytridiomycosis [53], although populations are in decline [55].

We found that a bacterium *F. johnsoniae* and a fungal probiotic *P. expansum* can increase the *Bd* killing function of the mucosome. The bacterium *P. fluorescens* did not show this effect. Because host AMPs did not appear to be affected by these treatments (Fig 3B), the observed effects are most likely caused by antifungal metabolites produced by the microbes growing on the amphibian skin [56]. Upregulation of host mucosal immunity excluding AMPs is an untested alternative mechanism, and potentially a beneficial host response to probiotics. A non-responsive immune system when given probiotics may be preferred from a conservation management standpoint in order for the probiotics to colonize the host, establish within the microbiota and persist. However, this is not necessarily common and immune stimulation in response to probiotics occurs in other systems [57,58].

An ideal probiotic would produce metabolites that inhibit *Bd* growth as shown above, and also be uninhibited by host skin defense peptides. A literature review demonstrates that skin peptides can inhibit the growth of some bacteria, but not others, and suggests that skin defense peptides may be critical in structuring the symbiotic community on amphibian skin [52]. Rollins-Smith *et al.* [35] showed that *Aeromonas hydrophila*, a common resident on amphibian skin and also an opportunistic pathogen, could tolerate high levels of host antimicrobial peptides. This organism shows antifungal characteristics including activity against *Bd* growth [33]. The ability of extracellular products of *A. hydrophila* to inhibit amphibian antimicrobial peptides indicates a co-evolutionary relationship between host and symbionts [59]. In addition, *Pseudomonas mirabilis* and *Serratia liquefaciens* were found to be resistant to antimicrobial peptides from several host frog species [60]. Here we used probiotics that largely resisted low concentrations of natural mixtures of host defense peptides (Fig S6 in File S1). Thus, to increase the likelihood of probiotic establishment, use of probiotics with a co-evolutionary relationship with the target host may be advantageous.

While easily cultured, the isolates tested here may not be dominant community members based on culture-independent analyses [31,61,62]. Therefore, future studies will benefit by examining the effects of probiotic treatments on the natural microbial communities on host amphibians using culture-independent techniques such as next-generation sequencing. While community interactions are difficult to test in vitro and before probiotics are applied to a host, our results affirm that testing probiotics under certain foreseeable contexts may increase the pace of biotherapy development.

Because potential probiotics that inhibit the growth of *Bd* only do so under certain conditions, we recommend the following screening criteria (Fig 5): (1) Candidates for probiotic development should be chosen from among the culturable microbiota locally present on tolerant hosts or populations that are able to persist with *Bd* [32,33]. (2) Candidates should have the capacity to inhibit *Bd* growth when grown in isolation, in co-culture with *Bd*, and in an environmental context relevant to the amphibian life-cycle, and (3) the ability to resist immune defenses on host skin, establish within the microbiota, and contribute to antifungal defenses in *vivo*. Resistance to mucosal immune defenses may be critical for establishment within the microbial community associated with the skin, and critical for long-term persistence. Some symbionts appear to be assisted in surviving on the host by thriving on skin mucosal products. Mucosal oligosaccharides, for example, differ among hosts and life-history stages, and may be a selective force in structuring the microbiota [63,64]. Amphibian skin provides a useful model of host-microbiota interactions to better understand mechanisms of microbial community assembly and maintenance within vertebrate mucosa. Indeed, these mechanisms underlie strategies to promote human health by manipulating microbial communities - a long-term goal of the Human Microbiome Project [7,65].

While screening for candidate probiotics, some beneficial organisms may be inadvertently discarded based on tests of bacterial filtrate on *Bd* growth. Microbes producing antifungal metabolites such as bacteriocins [66] or small molecule antibiotics [56,67] will be detected by this method. However, microbes may also compete directly for space or resources, and may exclude pathogenic fungi by other mechanisms [26,68]. Furthermore, microbial secondary metabolites such as prodigines produced by *Serratia* spp. can be immuno suppressive [67]. Probiotics may strongly influence host immunity through interactions with host Toll-like receptors or NOD-like receptors, or through interactions...
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Figure 5. Choosing probiotics with the greatest potential against amphibian chytridiomycosis. Candidate probiotic bacteria (or fungi) are isolated from populations of amphibians that are able to persist in the presence of B. dendrobatidis (Bd) [1]. To increase the chances of successful prophylactic biotherapy, candidate probiotics should be tested for at least three characteristics: (a) capacity to inhibit Bd growth as a pure isolate without specific competitive interactions to induce antifungal metabolites, (b) capacity to inhibit Bd at a temperature range consistent with host habitat, and (c) resistance to host skin immune defenses that would complicate probiotic establishment. Remedial biotherapy of already infected individuals should maintain antifungal capacity when grown in competition with Bd and withstand the sometimes lethal effects of Bd metabolites (Fig. S6 in File S1). Testing probiotic effect in vivo can be accomplished without resorting to pathogen exposure experiments by using the mucosome function assay described here. doi:10.1371/journal.pone.0096375.g005

with epithelial cells and immune system cells modulating both local and systemic immune responses [69]. The immunomodulatory effect of probiotics cannot be tested with in vitro Bd growth assays and host trials are necessary to test for these emergent properties of probiotics.

Antimicrobial peptides and a range of other defenses protect amphibian skin by synergizing or interacting with microbes [41,70]. Thus, a better indication of antifungal effect of probiotics was obtained by testing the mucosome directly on zoospore viability. In vitro screening cannot incorporate every factor and eventually in vivo trials, both in the lab and under natural conditions are necessary to determine if an overall health benefit is provided. However, beginning with a probiotic that is not likely to become an opportunistic pathogen with changing climatic conditions may be a consideration. Transmissible probiotics would aid disease control at the population level [33], and if able to persist through metamorphosis when applied to tadpoles, disease presentation at this critical developmental stage could be avoided for A. obstetricans and other susceptible amphibians [40]. Additionally, Bd metabolites are known to be toxic to amphibian lymphocytes [50], and in this study were toxic to certain bacteria such as Serratia plymuthica (Fig. S6 in File S1), perhaps prohibiting the use of certain probiotics intended as remedial biotherapy for infected individuals. The potential for negative biodiversity-function relationships, especially among mixtures of closely related bacteria, cautions against the use of probiotic mixtures that may cause interference competition and reduce host protection [71]. Further refinements to the probiotic screening and discovery process will incorporate next-generation sequencing analyses to target rare or as yet uncultured microbes of interest, and testing microbial consortia that appear linked to disease resistance function. Measuring the effectiveness of applied probiotics is a second step in managing disease risk.

No previous studies have attempted to relate skin microbiota or a holistic measure of skin defense function against Bd with disease susceptibility. Given the extreme complexity of the skin microecosystem and interactions described above, the holistic measure of mucosome function presents a significant advance in our capacity to predict relative disease susceptibility, and to measure the success of managed treatments without resorting to infection trials. Here, we examined overall prevalence of infection in Switzerland and Europe and test for correlations at these broad scales with innate defenses from selected life-stages and species (Fig. 1). We found a very strong correlation between mucosome function against Bd and infection prevalence in the field and upon experimental exposure. Since Bd-naïve amphibians were sampled for mucosome function, adaptive immunity such as mucosal antibodies is not indicated and antifungal function can be attributed primarily to innate defenses including the microbiota. Indeed, altering the microbiota through probiotic treatments affected mucosome function against Bd. In addition to assessing infection risk in natural amphibian assemblages, mucosome functional assays can now be used to assess risk in relict populations or in captive colonies slated for reintroduction. While the efficacies of human probiotics are under scrutiny [2], quantifying the effectiveness of amphibian probiotic treatments under scenarios of changing environmental conditions is a tangible goal.

Supporting Information

File S1 Protocol for determining Bd viability, supplementary tables and figures. (PDF)

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Author Contributions

Conceived and designed the experiments: DCW SB JK EK UT. Performed the experiments: DCW HB SB JK EK UT LRD CB SH. Analyzed the data: DCW SB JK EK UT BRS. Wrote the paper: DCW BRS RK VM. Performed field work: DCW JK UT LRD.

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