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REVIEW AND SYNTHESIS

Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use

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Abstract

Probiotic therapy through bioaugmentation is a feasible disease mitigation strategy based on growing evidence that microbes contribute to host defences of plants and animals. Amphibians are currently threatened by the rapid global spread of the pathogen, *Batrachochytrium dendrobatidis (Bd),* which causes the disease chytridiomycosis. Bioaugmentation of locally occurring protective bacteria on amphibians has mitigated this disease effectively in laboratory trials and one recent field trial. Areas still naïve to *Bd* provide an opportunity for conservationists to proactively implement probiotic strategies to prevent further amphibian declines. In areas where *Bd* is endemic, bioaugmentation can facilitate repatriation of susceptible amphibians currently maintained in assurance colonies. Here, we synthesise the current research in amphibian microbial ecology and bioaugmentation to identify characteristics of effective probiotics in relation to their interactions with *Bd*, their host, other resident microbes and the environment. To target at-risk species and amphibian communities, we develop sampling strategies and filtering protocols that result in probiotics that inhibit *Bd* under ecologically relevant conditions and persist on susceptible amphibians. This filtering tool can be used proactively to guide amphibian disease mitigation and can be extended to other taxa threatened by emerging infectious diseases.

Keywords

Amphibian, *Batrachochytrium dendrobatidis*, bioaugmentation, chytridiomycosis, disease mitigation, probiotic, wildlife diseases.

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INTRODUCTION

Microbial defences against pathogens are important for plants and animals (Berg 2009; Teplitski & Ritchie 2009; Gerritsen *et al.* 2011). Community structure is known to affect disease dynamics (Belden & Harris 2007; LoGiudice *et al.* 2008; Keesing *et al.* 2010), and evidence is accumulating that inter-specific interactions at the microbial level on individual hosts affect disease risk (Robinson *et al.* 2010; Grice & Segre 2011; Reid *et al.* 2011). Understanding interactions between pathogens and host microbes can enable us to manipulate microbial communities to improve health. Probiotic therapy through bioaugmentation is the augmentation of locally occurring protective bacteria to an individual or the environment with the purpose of altering the hosts' microbial community structure to mitigate disease (Haas & Défago 2005; Becker & Harris 2010; Gerritsen *et al.* 2011). Much research has targeted manipulation of microbiota in humans and aquacultural and agricultural species with positive and encouraging results (Fuller 1989; Verschuere *et al.* 2000; Kesarcodi-Watson *et al.* 2008). However, little research on disease mitigation using probiotics in nature has occurred despite the serious threats posed by emerging infectious diseases.

Amphibians are threatened by the fungal disease chytridiomycosis, which is associated with the dramatic declines or extinctions of over 200 amphibian species (Fisher *et al.* 2009; Kilpatrick *et al.* 2010). Chytridiomycosis is caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Longcore *et al.* 1999) and is the largest disease threat to biodiversity at the present time (Wake & Vredenburg 2008; Crawford *et al.* 2010). Its devastating effects likely are amplified by interactions with other anthropogenic threats (Collins & Storfer 2003). There are some areas with diverse amphibian assemblages that are currently *Bd*-free, such as Madagascar, which provide an opportunity to proactively prevent further catastrophic amphibian declines and extinctions. Susceptible individuals in survival assurance colonies are also in need of repatriation. For these reasons, a feasible disease mitigation strategy is imperative. Accumulating evidence suggests that probiotic strategies can be effective for amphibians, perhaps because probiotics extend the hosts' innate immune system (Harris *et al.* 2009a,b; Vredenburg *et al.* 2011; Myers *et al.* 2012; Rollins-Smith & Woodhams 2012). Furthermore, probiotic therapy research is elucidating principles of microbial ecology including establishment, transmission and temporal dynamics of host-associated microbiota. Here, we review and synthesise current amphibian microbial ecology and bioaugmentation research and use this synthesis to define characteristics of effective probiotics. Past probiotic choices for laboratory and field trials have been based on incomplete information and were driven by the urgent need to protect

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amphibian populations. Therefore, we also develop sampling strategies and filtering protocols to guide the selection of amphibian probiotics, which is essential for a proactive rather than a reactive approach to disease mitigation in amphibians and other wildlife species.

BACKGROUND AND GENERAL PRINCIPLES

Batrachochytrium dendrobatidis

Bd, a chytrid fungus, has two known life stages: a motile, flagellated zoospore and a sessile zoosporangium that resides in the amphibian epidermis (Berger *et al.* 2005). Released zoospores can either infect a new individual or re-infect the current host, meaning that infection and re-infection probability is a function of the hosts' defences, such as microbial defences, each generation. Periods of high host density, such as mating aggregations, can facilitate infection of new individuals (Kilpatrick *et al.* 2010). Several lineages of *Bd* have been identified. The hypervirulent global panzootic lineage is associated with massive declines and extinctions, and it tends to move in a wave (Lips *et al.* 2008; Farrer *et al.* 2011). Importantly, *Bd* persists in the environment due to some amphibians and perhaps other species acting as reservoirs (Reeder *et al.* 2012).

Amphibian immunity

Amphibian defences against *Bd* include acquired immunity, innate immunity and cutaneous microbial communities, and these defences likely interact (Rollins-Smith & Woodhams 2012). The robustness of amphibians' acquired immune response to *Bd* is debated (Rosenblum *et al.* 2009; Ramsey *et al.* 2010; Savage & Zamudio 2011) and is mounted slowly if it occurs at all (Rollins-Smith 2009). Down-regulation of immune system genes (Rosenblum *et al.* 2009) and ineffective vaccination attempts (Stice & Briggs 2010) suggest a poor acquired immune response. Innate immune activity provides a non-specific defence against cutaneous pathogens (Rollins-Smith 2009). Cutaneous antimicrobial peptide (AMP) production is a main component of amphibian innate immunity (Rollins-Smith 2009), but lysozyme and small organic molecules, such as alkaloids, also may play a role (Rollins-Smith & Woodhams 2012). Innate and acquired immunity protect some amphibian species from *Bd* (Woodhams *et al.* 2007a; Savage & Zamudio 2011); however, they offer little hope to naïve, susceptible species unless natural selection increases the frequency of individuals with these genetically based defences against *Bd*. In some cases, *Bd* has caused extinctions (Fisher *et al.* 2009), which strongly suggests that evolution of adequate defences is not universal.

Amphibian skin harbours symbiotic resident microbes, which constitute the only line of defence that is not directly host produced and has been successfully manipulated to mitigate disease (Harris *et al.* 2009a; Vredenburg *et al.* 2011). Antifungal cutaneous microbes have been cultured from every host species sampled, suggesting they can play a role against various pathogens (Lauer *et al.* 2007, 2008). Growing evidence supports the hypothesis that antifungal skin microbes suppress chytridiomycosis (Becker *et al.* 2009; Harris *et al.* 2009a,b; Vredenburg *et al.* 2011; Muletz *et al.* 2012). Bacteria inhibit *Bd* directly through production of inhibitory metabolites and perhaps indirectly through immunomodulation where microbes regulate the production of host defences such as AMPs and lysozyme (Reid *et al.* 2011). A bacteria removal experiment with *Plethodon cinereus* demonstrated that individuals with reduced microbiota had higher morbidity than individuals with an unmanipulated microbiota when exposed to *Bd* (Becker & Harris 2010). In complementary probiotic experiments, amphibians inoculated with an anti-*Bd* bacterium had reduced morbidity and mortality from *Bd* (Harris *et al.* 2009a,b), which was associated with the presence of a bacterially produced anti-*Bd* metabolite (Harris *et al.* 2009a). Importantly, a field experiment involving bioaugmentation of an anti-*Bd* species, *Janthinobacterium lividum,* on *Rana muscosa* in the Sierra Nevadas showed that frogs treated with probiotic baths had lower peak infection loads than untreated controls (Vredenburg *et al.* 2011). One year after treatment, untreated controls were not recovered whereas 39% of probiotic-treated individuals were recovered (Vredenburg, pers. comm.), suggesting that probiotic treatment allowed individuals to persist by preventing *Bd* from reaching a lethal threshold. Although continued optimisation of probiotic selection and protocols are needed, this research demonstrates that bioaugmentation has tremendous potential to assist vulnerable amphibian populations.

Amphibian microbial community ecology and probiotics

Microbial community establishment begins at hatching and can be strongly influenced by parental microbes, the pool of microbes in the environment and the interaction between the host's immune system and mucous composition and colonising microbes (Fierer *et al.* 2012). Mucopolysaccharides secreted by mucous glands likely provide the resources needed for bacterial growth, which initiates microbial competition. A recent model suggests that the assembly of a beneficial microbiome is dependent on interference competition where threshold densities within and among taxa trigger antimicrobial metabolite production (Scheuring & Yu 2012). Therefore, both microbe–host and microbe–microbe interactions will dictate community establishment.

Microbes can be transmitted vertically, horizontally and environmentally, and probiotic bacteria can be transmitted by these mechanisms. Vertical transmission likely occurs in amphibians with parental care. The frog *Hyalinobatrachium colymbiphyllum* appears to transfer a defensive microbiota to embryos, which likely protect hatchlings from *Bd* (Walke *et al.* 2011). If vertical transmission occurs, it could lead to probiotic transfer and persistence between generations. Horizontal transmission has not been investigated in amphibians, but likely occurs during mating and during congregations in winter hibernacula. If horizontal transmission occurs at a high rate, fewer amphibians will need probiotic treatment because treated individuals could transfer the probiotic to untreated individuals. Environmental transmission has been demonstrated with *Pl. cinereus*, where the probiotic *J. lividum* was transmitted from soil to salamanders in a laboratory experiment (Muletz *et al.* 2012). This result suggests that environmental transfer occurs in nature. If this exchange is frequent then environmental probiotic inoculation could be effective, as it would allow numerous amphibians to acquire the probiotic without individual treatment. Pseudo-environmental transmission occurs when bacteria from parents or other individuals are transferred to the environment and then to offspring or other amphibians. These modes of transmission are not mutually exclusive and likely work in tandem to shape amphibian microbial communities.

Knowledge of amphibian cutaneous microbial community structure is increasing. Next generation sequencing of the 16S rRNA gene provides more complete estimates of community structure and diversity than culturing studies (Grice & Segre 2011; McKenzie *et al.* 2011). Using 454 pyrosequencing, McKenzie *et al.* (2011) found that amphibian microbial communities tended to be species-specific rather than environment-specific and that levels of microbial diversity differed among amphibian species. In addition, in several studies, a few microbial taxa were found across amphibian species and locations (Lauer *et al.* 2007, 2008; Woodhams *et al.* 2007b; Lam *et al.* 2010; McKenzie *et al.* 2011), suggesting some cutaneous symbionts have a broad host range.

Cutaneous microbial community structure and its stability are likely associated with disease outcome. In humans, it is not clear whether a certain community structure leads to disease or is a consequence of disease (Grice & Segre 2011); however, in amphibians, experimental studies show that altering their microbial community affects disease susceptibility (Harris *et al.* 2009b; Becker & Harris 2010). The stability of amphibian cutaneous microbial communities is linked to microbial maintenance. Microbes can be maintained after disturbances such as skin sloughing through environmental reinoculation or from bacterial reservoirs on the host (Meyer *et al.* 2012; Muletz *et al.* 2012). In *Pl. cinereus*, concentrations of bacteria were found in gland openings (Lauer *et al.* 2007) that may provide a "seed bank" from which microbes can repopulate the skin. The rate of skin sloughing and microbial repopulation likely influences disease risk (Myers *et al.* 2012). At metamorphosis, the microbial community of aquatic larvae may shift to adjust to terrestrial conditions and changing host immunity, and this could cause a period of instability that affects disease susceptibility.

Microbial community structure likely fluctuates, but an important question is whether community function, including defensive function, will remain constant (Fierer *et al.* 2012; Huttenhower *et al.* 2012). Defensive function on amphibian skin can be assayed by determining the relative abundance of anti-*Bd* bacterial metabolites using HPLC-MS (Brucker *et al.* 2008a) or with total cutaneous molecule bioassays (Box 2). For defensive function, the specific bacterium may not be important, but rather the genes it carries. Horizontal gene transfer (HGT) plays a role in functional stability in the human gut microbiome (Smillie *et al.* 2011). Probiotic species that have anti-*Bd* metabolite genes on plasmids could readily pass these genes to other community members and contribute to functional stability (Robinson *et al.* 2010). The potential role of HGT in amphibian microbial communities and defensive function is unexplored. With a better understanding of community structure and functional stability and its relationship to chytridiomycosis, disease susceptibility can be predicted and interventions to establish protective microbial communities can be implemented.

Ecosystem function, including resistance to pathogen invasion, can improve as species diversity increases (Balvanera *et al.* 2006; van Elsas *et al.* 2012; Fig. 1), but it is also possible that the role of individual species trumps diversity (Lyons *et al.* 2005; Box 1). Few studies have used controlled experiments to relate microbial community diversity to defensive function. One experiment demonstrated that higher locust gut microbial diversity increased disease resistance (Dillon *et al.* 2005). Preliminary evidence from Australian Wet Tropics frogs indicates that *Bd* infection intensity is negatively correlated with anti-*Bd* bacterial richness, suggesting a possible role of diversity in defensive function. Alternatively, an individual species can provide a disproportionate share of the community's defensive function. To date, experimental evidence suggests that the addition of one probiotic species can increase defensive function (Becker *et al.* 2009; Harris *et al.* 2009a,b; Muletz *et al.* 2012). The degree to which diversity and key bacterial species provide defence against disease will dictate bioaugmentation strategies (Box 1).

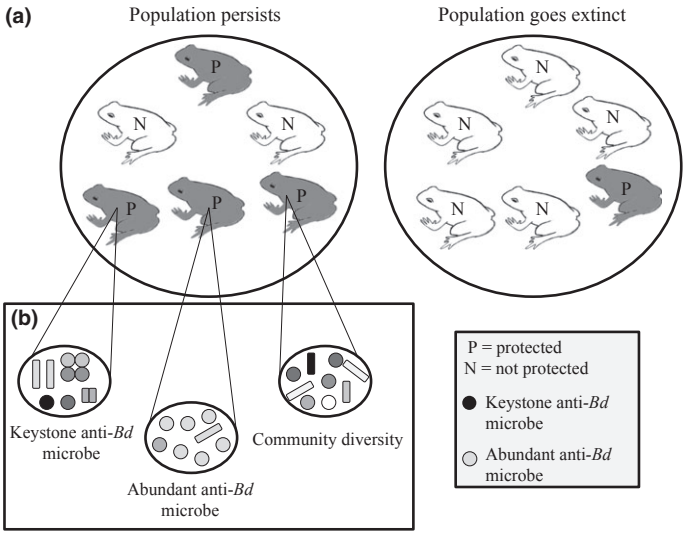

Figure 1 Population and community mechanisms of protection from *Bd*. (a) Herd effect in which a population persists with *Bd* because a large proportion of the individuals are protected by beneficial microbes (left), whereas a population goes extinct when a low proportion of the individuals are protected (right). (b) Individuals are protected by one of three possible mechanisms: a keystone anti-*Bd* microbe restructures the cutaneous microbial community into one that is stable and provides increased defensive function, an abundant anti-*Bd* microbe provides a major portion of the defensive function, or a high level of microbial diversity is associated with defensive function. A goal of probiotic therapy is to increase the proportion of protected individuals in populations via one of these mechanisms thereby allowing the population to persist with the pathogen. Shaded frogs indicate protected individuals.

Bacterial-host evolution

Selection at the individual microbe, individual amphibian and amphibian population levels are expected following exposure to *Bd*. As *Bd* invades amphibian skin, selection for anti-*Bd* microbial genotypes should occur. Amphibian hosts in a population have different microbial communities that can vary in defence against *Bd*, and individuals with a more protective microbiota will have a higher fitness. In addition, *Bd*-naive populations are likely to differ in the proportion of individuals with protective microbial species (Lam *et al.* 2010). Populations with a high proportion of individuals with a protective microbiota appear to benefit from an analogue of herd immunity, in which an infectious disease dies out when the proportion of immunised or protected individuals is above a threshold value (Woodhams *et al.* 2007b; Lam *et al.* 2010). Therefore, one goal of probiotic therapy is to increase the proportion of individuals in a population that have protective microbes (Fig. 1). It is important to note that the pathogen is expected to evolve resistance to host and microbial defences and that evolution of host microbial defences is

faster than evolution of host-produced defences because of the shorter generation time of microbes.

Microbial community structure may be heritable and therefore can respond to selection. Vertical and pseudo-environmental transmission are possible mechanisms of resident microbe heritability. In addition, amphibian AMPs and antibodies likely are important forces structuring amphibian microbial communities, as they are in humans (Grice & Segre 2011; Gallo & Hooper 2012). Genetically based differences in immune system characteristics can therefore lead to different and heritable community structures even without vertical transmission if environmental reservoirs are available. Evidence of a genetic component to microbial community structure in human intestinal tracts has been detected (Zoetendal *et al.* 2001), although recent studies have found low heritability (Yatsunenko *et al.* 2012). Knowledge of the heritability of amphibian microbial communities is lacking but will be important for understanding community dynamics and its evolution.

CHARACTERISTICS FOR AN EFFECTIVE PROBIOTIC AGAINST CHYTRIDIOMYCOSIS

Understanding the ecological interactions that govern microbial community dynamics on amphibian skin is key to defining effective probiotic characteristics. The primary roles of a probiotic are to prevent *Bd* from colonising amphibian skin and to prevent minor infections from escalating to a lethal threshold (Vredenburg *et al.* 2010). These roles, along with the probiotic candidates' interactions with the host, the cutaneous microbial community and the environment, are important and must be considered when selecting bacteria for bioaugmentation strategies.

Box 1 Keystone-probiotic hypothesis

Recently, Hajishengallis *et al.* (2012) developed a keystone-pathogen hypothesis stating that microbial pathogens in low abundance can dictate outcomes of certain diseases, such as periodontal disease or inflammatory bowel diseases, by restructuring the normal microbiota into a dysbiotic state. Certain immunopathologies, chronic diseases, and cancers have also been posited with this trigger (Ewald 2010). This differs from the traditional understanding that the microbial pathogen causes disease, in part, by increasing in abundance (Hajishengallis *et al.* 2012). Our current hypothesis is that a probiotic protects an amphibian by becoming an abundant member of the cutaneous community and by producing anti-*Bd* metabolites (Brucker *et al.* 2008a,b; Becker *et al.* 2009). For example, Becker *et al.* (2009) found an association between the concentration of violacein, a bacterially produced anti-*Bd* metabolite, and survival of salamanders when exposed to *Bd*. The concentration of violacein was correlated with *J. lividum* abundance, which is the bacteria that produces it, suggesting that a more abundant probiotic will be more protective.

We use a similar framework to develop a keystone-probiotic hypothesis. A keystone-probiotic will be in low abundance, have a significant impact on community structure leading to a greater defensive function, and may not itself provide a health benefit but causes changes in the microbial community that benefit host health. The value of a keystone-probiotic is that it remodels the cutaneous microbial community into one that is stable and provides increased defensive function (Fig. 1). Community restructuring can be caused by the keystone-probiotic in several ways. The probiotic can stimulate host immune defences, such as AMP production, that can differentially affect microbial community members. Host-produced nutrients in mucosal secretions can also be manipulated by keystone-symbionts; for example, *Bacteroides thetaiotaomicron* help stabilise gut microbiota by inducing gut epithelial cells to produce fucosylated glycans (Bäckhed *et al.* 2005). Furthermore, the probiotic might differentially affect an important competitor in the microbial food web and either increase or decrease its population density and therefore lead to an altered community structure. If the resulting community inhibits pathogens by mechanisms such as increased anti-*Bd* metabolite production or spatial competition, then the probiotic has a keystone effect. The probiotic also might have a disproportionate effect by acting synergistically with resident members of the microbiota to inhibit *Bd*. For example, the probiotic might induce resident microbes to produce anti-*Bd* metabolites. Importantly, a keystone-probiotic must bring about a stable community, minimise pathogen colonisation, and prevent pathogen densities from increasing. At this point, it is not known whether the success of probiotic manipulations is due to direct effects of the bacterial species producing anti-*Bd* metabolites, indirect effects of community restructuring, synergistic interactions with resident microbes, induction of host responses or a combination of these mechanisms.

These two alternative models – a probiotic that becomes abundant and a keystone-probiotic that has large effects through community restructuring while remaining relatively rare – have implications for disease mitigation. Species that are relatively rare are subject to stochastic loss, especially during disturbances such as skin sloughing (Meyer *et al.* 2012). Thus, a keystone-probiotic may need continual replenishment via environmental transmission. Both models predict pathogen protection with at least some community restructuring, so an important criterion is stability of the new protective community.

Next generation sequencing data can lead to mechanistic insights. It is possible to correlate individual species' abundances with community structure, and this analysis will predict which species dictate characteristic community structures. These community structures also can be correlated with disease outcomes, and therefore a correlation between keystone species and disease outcome can be determined. Survey data can suggest which bacteria will be effective probiotic candidates regardless of whether they are relatively rare or relatively abundant on amphibians. If these species are culturable, they can be tested with the filtering protocols presented in the text. Of course, experimental manipulations, which are feasible with amphibian hosts, are necessary to determine cause and effect. For example, experiments can be conducted where a potential keystone-probiotic is added to a variety of resident microbial community structures and response to *Bd* infection can be measured. These experiments can determine the roles of keystone species, microbial diversity and their interaction leading to protection from *Bd*.

Probiotic-*Bd* interactions

Interactions between *Bd* and the probiotic are a primary factor influencing a probiotic's ability to repel and inhibit *Bd* (Table 1). *Bd* requires space and nutrients to colonise, grow and develop. High densities of resident bacteria can limit attachment sites and available nutrients for *Bd* (Collado *et al.* 2008; Mohapatra *et al.* 2012), thereby decreasing zoospore colonisation and slowing development. Effective probiotic therapy requires that the probiotic bacteria be an effective competitor and colonise and persist on the skin, especially in regions that are typically infected by *Bd*, such as the ventral surface, limbs and feet (North & Alford 2008). Bacterial spatial distributions have not been investigated, but could be determined by sampling different body regions or visualising bacteria with fluorescence microscopy. With this knowledge, candidate probiotics could be graded on where and how densely they colonise and persist on amphibians.

Bacterially-produced metabolites are responsible for both repelling and inhibiting *Bd*. We have evidence that two metabolites (2,4-diacetylphloroglucinol [2,4-DAPG] and indole-3-carboxaldehyde [I3C], Fig. 2) repel *Bd* in laboratory assays (Lam *et al.* 2011). This negative chemotaxis may prevent colonisation or re-colonisation of zoospores discharged from zoosporangia. Bacterial metabolites (violacein, 2,4-DAPG, I3C) also inhibit *Bd* growth in *in vitro* bioassays (Brucker *et al.* 2008a,b). Importantly, metabolites were detected on amphibian skins in nature at concentrations that were inhibitory in laboratory assays (Brucker *et al.* 2008b).

Two bacterial addition experiments, where individuals were immersed in a probiotic bath, demonstrated a strong association between violacein concentration and survival after *Bd* exposure. In the first experiment, addition of the violacein producer, *J. lividum,* to *R. muscosa* led to significantly higher violacein concentrations on skins and higher survival compared with untreated controls (Harris *et al.* 2009a). In the second experiment, a threshold level of violacein on *Pl. cinereus* was associated with survival (Becker *et al.* 2009).

Microbial defences are likely achieved as a by-product of inter- and intra-specific microbial competition. Quorum sensing, a process of cell-to-cell communication where a threshold population density regulates gene expression, can trigger metabolite production. Bacterial species vary in their threshold densities (Mohapatra *et al.* 2012); therefore, an effective probiotic will produce anti-*Bd* metabolites at fairly low cell densities or will grow rapidly to reach a density where metabolites are produced. A fast-acting and stable defence is likely to be dependent on rapid metabolite production upon initial colonisation and re-colonisation after cutaneous disturbances. Furthermore, a probiotic must maintain its defensive function in *Bd*'s presence. Studies in our laboratories have shown that some bacteria, when co-cultured with *Bd,* are later unable to inhibit *Bd*, suggesting *Bd* possesses a mechanism that down-regulates bacterial metabolite production. Such species would not be appropriate as probiotics. Research has focused on detection of small organic metabolites, but bacterially produced defensive peptides such as bacteriocins, documented in human skin microbiota (Gallo & Hooper 2012), also warrant investigation.

Table 1 Summary of the characteristics of effective probiotics based on four interactions

	Characteristics of effective probiotics
Probiotic-*Bd*	Repel and inhibit *Bd*
	Maintain defensive function in presence of *Bd*
Probiotic-resident microbial community	Coexist with functionally important bacterial species
	Positively interact with resident bacteria
	Shift microbial community to a defensive state (Box 1)
Probiotic-host	Colonise and persist on host
	Positively interact with host-produced defences
	Do no harm to host
Probiotic-environment	Inhibit *Bd* under appropriate ecological contexts
	Have minimal non-target effects
	Form a self-disseminating system between amphibian and the environment

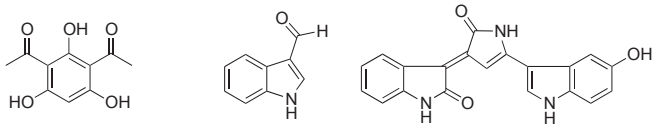

Figure 2 Three bacterially-produced metabolites found to inhibit *Bd* growth.

Probiotic-host interactions

A probiotic's interactions with its host will determine its effectiveness at inhibiting *Bd* and decreasing *Bd*-associated morbidity and mortality (Table 1). In order for probiotic bacteria to colonise and persist successfully, they must use cutaneous resources provided by the host or resident microbes and not be inhibited by host immune defences. Strong evidence indicates that anti-*Bd* bacteria species, such as *Pseudomonas fluorescens*, are not inhibited by moderate to low concentrations of amphibian AMP mixtures (Myers *et al.* 2012), but this situation is not universal (Schadich & Cole 2010). Certain symbiotic bacteria can induce AMP production through immunomodulation (Grice & Segre 2011; Reid *et al.* 2011; Mohapatra *et al.* 2012); therefore, probiotic bioaugmentation may trigger production of *Bd*-inhibitory AMPs (Rollins-Smith & Woodhams 2012). Alternatively, microbes can reduce the need for costly peptide production. For example, Woodhams *et al.* (2012a) found that AMP production increased in response to *Bd* infection only when microbial defences were experimentally reduced.

A successful probiotic will not be pathogenic to the host or trigger a negative reaction from the innate or acquired immune system. The period after an amphibian hatches is likely a critical time of microbial community establishment when the acquired immune system adjusts to a resident microbiota. Treating larvae and hatchlings with a probiotic may increase the chances of acceptance by the host immune system and allow persistence. In aquaculture, treatment of larval stages often leads to increases in survival compared to control treatments (Nogami & Maeda 1992; Kesarcodi-Watson *et al.* 2008).

For later stages, augmenting a probiotic already found on the host species will be less likely to trigger a negative host reaction.

An effective probiotic will work additively or synergistically with host AMPs. The metabolite 2,4-DAPG, produced by *Ps. fluorescens*, worked synergistically *in vitro* with the *R. muscosa* AMP mixtures to inhibit *Bd*, meaning that relatively low concentrations of each molecule were required for inhibition when they occurred together. Importantly, *Ps. fluorescens* was not killed by AMP concentrations needed for synergistic inhibition (Myers *et al.* 2012). This interaction with host immunity suggests the basis of a mutualism between amphibian hosts and their bacterial symbionts.

Probiotic-resident microbial community interactions

A probiotic's interaction with resident skin microbiota will influence its efficacy (Table 1, Box 1). A probiotic should not eliminate functionally important resident bacteria. In addition, it may be important to select a probiotic that synergises with resident microbes. We have preliminary evidence from *in vitro* assays that four inhibitory bacteria species collected from *Pl. cinereus* work additively or synergistically to inhibit *Bd* when their culture filtrates are mixed in pair-wise combinations. Further work is necessary to see if bacterial synergies occur *in vivo*.

Probiotic-environment interactions

The environmental context in which a probiotic will be used must be considered, as it will affect its defensive function (Table 1). For example, temperature affects the pathogen, host and cutaneous microbes (Rohr & Raffel 2010; Daskin & Alford 2012). Amphibian immune function has an optimal temperature range that may not correspond to the optimal temperature range for *Bd* growth (17–25 °C) (Piotrowski *et al.* 2004; Woodhams *et al.* 2008). *Bd* prevalence is greater in cooler seasons in temperate ecosystems and montane tropical regions (Raffel *et al.* 2006), which could be a function of reduced host defences. Ideally, an effective probiotic will compensate by functioning outside of the optimal temperature range of host immunity. Furthermore, it is important to eliminate a bacterial species that inhibits *Bd* at one temperature, but facilitates it at another. An ideal probiotic should maintain its defensive function over an ecologically relevant temperature range.

Preventing *Bd*-associated population collapses and successfully repatriating amphibians from assurance colonies can be aided if the probiotic forms a self-disseminating system between the amphibian and the environment (Muletz *et al.* 2012). Amphibians likely obtain their microbiota from the environment at some point during development. This transfer from the environment may occur continually,

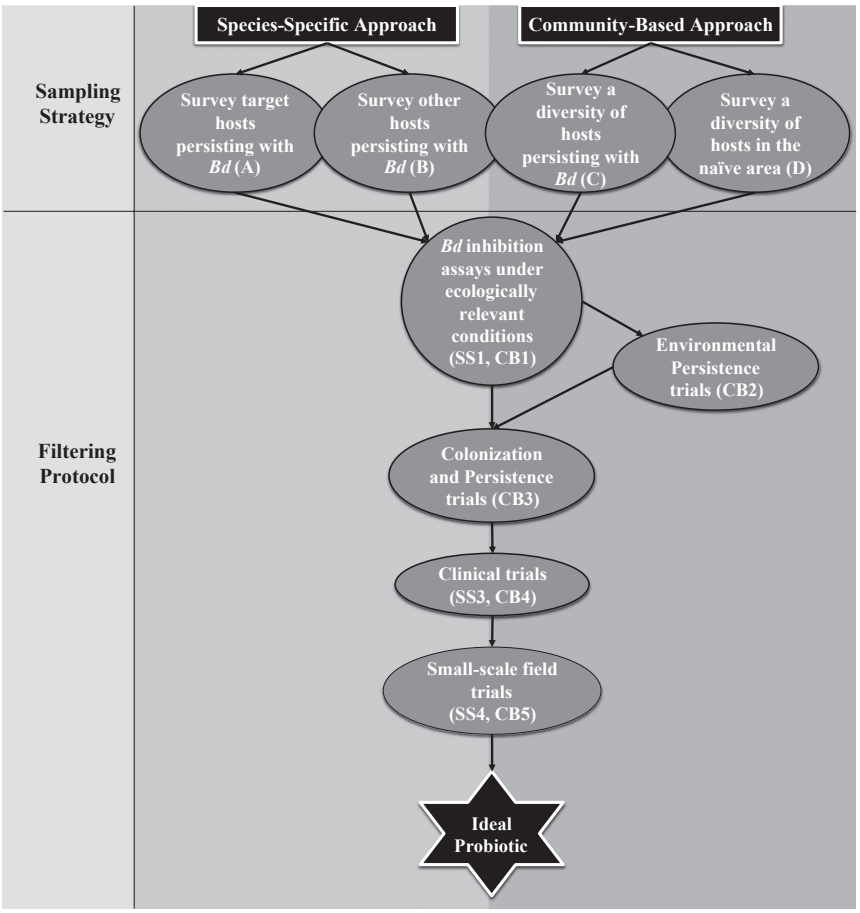

Figure 3 Sampling strategies and filtering processes for the selection of species-specific and community-based probiotics. Notations in parentheses link the elements of the figure to expanded discussion in the text. SS = species-specific; CB = community based.

making persistence of microbial communities and probiotics in part dependent on environmental sources. If so, the need for bioaugmentation could be linked to changes in microbial communities in soil and water due to factors such as climate change and pesticide plumes (Belden & Harris 2007). Laboratory trials have demonstrated environmental transmission (Muletz *et al.* 2012). However, studies are urgently needed to determine whether broad-scale probiotic environmental inoculation can be transmitted to amphibians and confer defence against *Bd*, whether environmental inoculation can lead to a self-disseminating system, and whether non-target ecosystem effects occur. Some agricultural studies suggest that broad-scale inoculations can be safe and effective (Scherwinski *et al.* 2008), but careful testing to ensure biosafety is necessary.

SPECIFIC RECOMMENDATIONS

The range of interactions among *Bd*, skin bacteria, host and environment leads us to propose sampling strategies and filtering protocols that are designed to guide selection of effective probiotics for protecting individual species and amphibian communities. The filtering protocol differs from listing effective probiotic characteristics as presented in other studies (Fuller 1989; Kesarcodi-Watson *et al.* 2008). Isolates are placed through a series of tests that progressively filter out ineffective ones, leaving the most promising candidates. A species-specific approach focuses on treating at-risk individuals with probiotic baths while a community-based approach targets amphibian assemblages by treating ponds or local areas with a broad-spectrum probiotic. We stress that bioaugmentation approaches must use microbes found in the local environment to improve success and minimise biosafety concerns.

Species-specific approach

Species-specific probiotics should target individuals being repatriated from survival assurance colonies (Becker *et al.* 2011) and individuals of critically endangered species in front of an advancing *Bd* wave (Woodhams *et al.* 2007b; Vredenburg *et al.* 2010). Assurance colonies have been implemented to rescue species when they are experiencing rapid declines across their range or when there is an imminent threat to amphibian populations due to the anticipated arrival of *Bd*. The goal of assurance colonies is to reintroduce threatened species to their native habitats and establish persisting populations; however, releasing susceptible individuals will be unsuccessful because *Bd* persists in the natural environment on reservoir species (Reeder *et al.* 2012). In addition, there are situations where susceptible species in front of an advancing *Bd* wave in the wild are not in assurance colonies (Vredenburg *et al.* 2010). In both cases, individuals can be treated with a probiotic derived from the appropriate sampling strategy and that successfully passes through the filtering protocol outlined below.

The sampling strategy for obtaining a species-specific probiotic for assurance colony species and endangered wild species will differ for species that have some populations in the wild coexisting with *Bd* (e.g. *Anaxyrus boreas* and *R. muscosa*), and species that are extirpated from the wild (e.g. *Atelopus zeteki*). If there are populations coexisting with *Bd*, it is essential to sample and culture microbes from members of these populations (Fig. 3a). For species that have been extirpated from the wild, it will be necessary to focus microbe sampling on related species that have a similar life-history, are found in similar habitats and locations, and are coexisting with *Bd* (Fig. 3b). Individuals in populations coexisting with *Bd* are surviving with *Bd* infection and are more likely to have anti-*Bd* bacteria.

Once microbes are collected from amphibians using standard methods (Harris *et al.* 2006), they must pass through the filtering criteria, which leaves a progressively smaller number of probiotic candidates. The candidate probiotics must inhibit *Bd* under ecologically relevant conditions of the intended host (Fig. 3 (SS1), Box 2). For example, it is essential that probiotics inhibit at temperatures at which the amphibian is most vulnerable to *Bd* infection (Daskin & Alford 2012). Preference should be given to inhibitory isolates that are present on a large proportion of sampled individuals since ubiquity suggests the isolate will persist on the target amphibians. The remaining candidate probiotics must colonise and persist on target amphibians at all life-history stages while not harming the host (Fig. 3 (SS2), Box 2). If bacteria are collected from surviving individuals of the intended host, the likelihood of bacterial persistence is high. If persistence is observed, it indicates that the host's immune system or resident microbes do not inhibit the isolate. It will be important to eliminate isolates that inhibit *Bd in vitro*, but do not persist or provide continual inhibition of *Bd* on amphibians (Box 2). The remaining candidates must inhibit *Bd* in clinical trials with all life-history stages to confirm *in vivo* effectiveness of the candidate probiotic to prevent disease (Fig. 3 (SS3), Box 2). Successful probiotics will decrease mortality and sub-lethal effects for all stages. Lastly, selected isolates must inhibit *Bd* in a small-scale field trial to assess effectiveness in the natural environment (Fig. 3(SS4), Box 2). At this point, remaining candidates have a high likelihood of being effective probiotics for the target amphibians.

Two amphibian species currently established in assurance colonies, the boreal toad, *An. boreas,* and the Panamanian golden frog (*At. zeteki*), are targets for pre-release probiotic treatment. The toad *An. boreas* is a species that has experienced population declines (Muths *et al.* 2003); however, there are wild populations persisting with *Bd* infection that should be sampled to obtain probiotic candidates (Fig. 3a). The collected microbes should be screened through the four-step filter discussed above. *At. zeteki* is a species that is likely extirpated from the wild. Becker *et al.* (2011) tested a probiotic candidate, *J. lividum*, on *At. zeteki*, which was isolated from North American salamanders. The probiotic treatment kept infection loads low initially, but the probiotic abundance declined and mortality occurred. Subsequently, 600 isolates were collected from related species coexisting with *Bd* in the same locations and habitats where *At. zeteki* was found and are currently being screened using the criteria listed above. Importantly, inhibition trials (step 1) removed 85% of isolates from consideration as a probiotic (Fig. 3b).

The susceptible frog in the Sierra Nevadas, *R. muscosa,* is an example of a species where populations in front of an advancing *Bd* wave are in need of protection. Probiotic candidates have been collected from populations that have persisted through the arrival of *Bd* and are therefore more likely to possess *Bd*-inhibitory bacteria (Fig. 3a) (Woodhams *et al.* 2007b; Lam *et al.* 2010). One *R. muscosa* population under threat from imminent *Bd* arrival, and predicted to be decimated, provided an opportunity for probiotic application. Due to the short lead-in time available, it was not possible to apply all elements of the filtering process. However, the probiotic *J. lividum* was chosen due to its success in previous experiments (Harris *et al.* 2009a) and its presence on a number of amphibian species

across many locations, including the Sierra Nevadas. This trial was successful: greater survival was seen for treated individuals, and *Bd* loads remained low compared to untreated controls (Vredenburg *et al.* 2011). Therefore, when immediate treatment is necessary, and little time exists for a full filtering process, priority can be given to probiotics that have been successful in other studies, assuming that

Box 2 Methodologies of filtering protocol

Inhibition assays

To determine the inhibitory nature of the candidate probiotics, we advocate the following protocols. The bacterial isolate should be co-cultured with *Bd*, because it will induce the bacteria to produce anti-*Bd* metabolites. In addition, isolates that are inhibited by *Bd* will be excluded. The culture filtrate (cell-free supernatant) that includes bacterial metabolites from the co-culture is assayed for *Bd* inhibition in 96-well microtiter plates (Bell *et al.* 2013). A negative control (heat-killed *Bd*), positive control (*Bd* without culture filtrate but with the equivalent volume of medium) and a control for *Bd*-produced metabolites (culture filtrate from a *Bd* culture) should be included. Inhibition assays can also be carried out on agar plates (Harris *et al.* 2006). In this protocol, *Bd* is spread evenly across the tryptone-agar plate and bacteria are streaked across the *Bd*-covered plate. After 72–96 h of incubation, the inhibition zone is measured. Trials should be replicated to accurately estimate inhibition and allow for statistical tests.

Colonisation & persistence trials

To assess colonisation and persistence, candidate probiotics are inoculated onto amphibians of all life-history stages in laboratory trials. For species-specific treatment strategies, amphibians are bathed in probiotic baths; for community treatment strategies, the housing substrate is inoculated with the probiotic. Colonisation and persistence can be assessed using culture-based or molecular methods (Becker *et al.* 2011). If culture-based techniques are used, artificial selection of bacterial isolates for rifampicin resistance can facilitate tracking during experiments (Muletz *et al.* 2012). For molecular detection, polymerase chain reaction (PCR) can be used to confirm colonisation and persistence of the probiotic. This technique requires the use of species-specific primers, which have been developed for some species such as *J. lividum* (Harris *et al.* 2009a). In all experiments, control groups of untreated amphibians are required. Ideally, during these trials swabbing or bathing should be used to periodically collect amphibian skin secretions, which are a mixture of defensive products of amphibians and their microbial symbionts. This protocol is currently being optimised. These secretions are used in *Bd* inhibition assays to compare control treatments (no probiotic) to probiotic treatments as a measure of the probiotic's *in vivo* effectiveness against *Bd*. Because these bioassays assess *in vivo* effectiveness of potential probiotics, they reduce the possibility of unsuccessful clinical trials.

Environmental persistence trials

Probiotic persistence in the environment is determined through laboratory trials where an environmental substrate is inoculated with the probiotic candidate (Muletz *et al.* 2012) and monitored over time. Depending on the habitat of the intended hosts, trials are conducted with water or soil as the substrate. Probiotic transmission can also be assessed if amphibians are housed in the inoculated substrate. Transfer of the probiotic to the host and persistence in the environment can be measured using culture-based or molecular methods (Becker *et al.* 2009; Muletz *et al.* 2012). A similar protocol can be used for trials conducted in nature.

Clinical trials

Laboratory-based clinical trials for species-specific probiotic treatment involve bathing amphibians in the probiotic and exposing both treated individuals and untreated controls to *Bd* in randomised, replicated trials (Harris *et al.* 2009a). Clinical trials for community-based probiotics involve inoculating the laboratory environment (water or soil) with the candidate probiotic and housing the selected host amphibians in these treated environments as well as housing a set of individuals in untreated control environments. Amphibians in both treatments should be exposed to *Bd* and monitored for survival and sublethal effects (i.e. growth rate, behaviours) (Harris *et al.* 2009a,b). Estimating *Bd* loads via qPCR (Hyatt *et al.* 2007) can be helpful in determining whether the probiotic kept *Bd* loads below a lethal threshold. These trials need to be replicated and conducted under ecologically relevant conditions. In addition, they should be conducted on all life-history stages, (i.e. larvae, juvenile, adult) to ensure the probiotic is effective across all stages.

Field trials

Small-scale probiotic field trials should be completed at locations where appropriate regulatory approval has been obtained. For species-specific strategies, field trials involve treatment of individuals with and without a probiotic bath and release at the field location (Vredenburg *et al.* 2011). Monitoring of *Bd* infection, the establishment of the probiotic on amphibians and ultimately the survival of released individuals will determine the outcome of the experiment. Field trials for community-based environmental treatment involve inoculation of soil or water with a probiotic and release of amphibians to treated areas. Survival of amphibians at the treated sites, *Bd* loads and probiotic abundance on the hosts and in the environment should be monitored and compared to control sites to evaluate success.

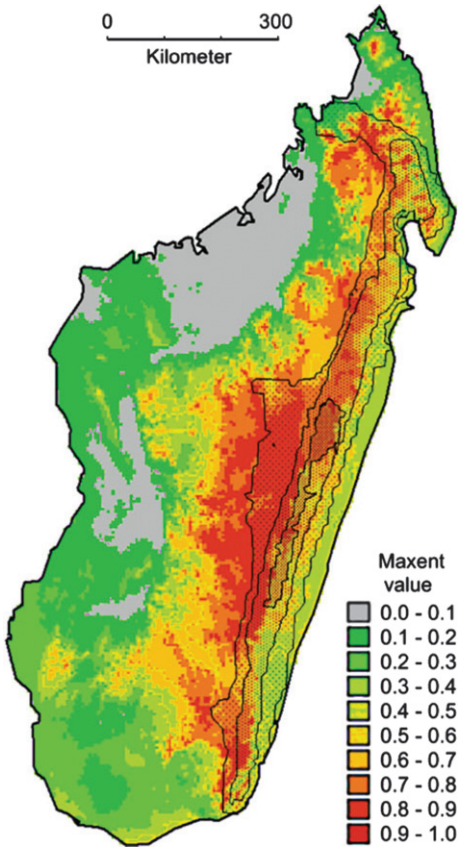

Figure 4 Potential distribution of the amphibian chytrid fungus in Madagascar following the ecological-niche modeling presented in Rödder *et al.* (2009). Warmer colours indicate a higher climatic suitability for *Bd* and are areas where amphibian endemism and diversity are high.

a strain of the probiotic can be found on amphibians in the intended application area.

COMMUNITY-BASED APPROACH

There are large areas with diverse amphibian assemblages, such as Madagascar, that remain *Bd*-free; however, its arrival is inevitable (Fisher & Farrer 2011; Box 3). Ecological niche modelling predicts suitable habitat for *Bd* in regions of Madagascar where the amphibian diversity is the greatest (Andreone *et al.* 2005; Rödder *et al.* 2009; Fig. 4). In this situation, the opportunity exists to be proactive and prevent the loss of many amphibian species. Probiotic conservation approaches for naïve communities differ from species-specific conservation efforts. The goal for community treatment is not to have a probiotic specific to one amphibian species but to have one or more probiotics that are suitable for multiple amphibian hosts. Importantly, certain anti-*Bd* species can exist on a number of diverse host species. For example, the probiotic species, *J. lividum*, has been found on two species of plethodontid salamanders in Virginia, on one species of high-altitude pond-dwelling frogs in California, on one species of low elevation frogs in Switzerland and on three species of high-altitude rainforest frogs in Ecuador (Lauer *et al.* 2007, 2008; Woodhams *et al.* 2007b). Certain anti-*Bd* genera, such as *Pseudomonas*, also have been commonly found on amphibians (Lauer *et al.* 2007, 2008; Walke *et al.* 2011), suggesting that these taxa could be good community probiotic (Harris *et al.* 2009b). The genus *Curvibacter* has also been found on multiple amphibian species (McKenzie *et al.* 2011). If it inhibits *Bd*, it could act as a broad-spectrum antifungal probiotic, just as commercially available agricultural probiotics have a broad host range (Berg 2009). Community-based treatment ideally would occur through environmental bioaugmentation, where one or a few environmental inoculations would allow numerous amphibian species as well as both larval and adult stages to be treated without individual capture.

The sampling strategy for obtaining a community-based probiotic will differ for amphibian communities that have neighbouring communities persisting with *Bd* [e.g. areas in Panama (Fig. 3c) (M. Hughey, pers. comm.)] and those that do not [e.g. Madagascar (Fig. 3d)]. In the first case, a wave of *Bd* is moving forward, but there are areas that remain *Bd*-free in addition to areas behind the wave that are now persisting despite infection. Bacteria that have a high prevalence on species persisting with *Bd* are more likely to be inhibitory and should be sampled to find effective probiotics (Fig. 3c). Some large geographical areas, such as Madagascar, are *Bd*-free to date and there are no amphibians coexisting with *Bd* from which to collect bacteria (Box 3). Under this scenario, broad-scale microbial surveys of amphibians need to be completed proactively (Fig. 3d).

After culturing the collected bacteria, they must pass a series of filtering criteria, similar to that described above. Candidates must inhibit *Bd* under ecologically relevant conditions representative of the intended community (Fig. 3 (CB1), Box 2). Preference should be given to inhibitory isolates that are found on a high proportion of species. The remaining candidates must persist in environmental conditions, such as temperature and pH, representative of the target application area (Fig. 3 (CB2), Box 2). Those that do so must also colonise and persist on hosts via environmental transmission (Muletz *et al.* 2012) (Fig. 3 (CB3), Box 2). Candidates that remain must reduce infection and the effects of chytridiomycosis in randomised, replicated clinical trials (Fig. 3 (CB4), Box 2). Finally, remaining isolates must maintain their effectiveness on amphibians in the natural environment (Fig. 3 (CB5), Box 2). All trials involving host amphibians should be conducted on a sample of phylogenetically diverse host species and all life-history stages under ecologically relevant conditions. The isolates that make it through this filtering process will likely be strong probiotics for community-based treatment. Finding effective community-based probiotics will require a concerted effort; however, the potential benefits in terms of preventing amphibian extinctions are substantial. As we learn more about mechanisms of inhibition as a function of probiotic species, the host and community context, the filtering criteria can be optimised for both species-specific and community-based treatment modalities.

APPLICATION OF PROBIOTIC BACTERIA

Optimisation of protocols for probiotic application is essential. There are two ways to apply probiotics: individual treatment and environmental treatment. For individual probiotic bath treatment, colonisation success could be increased by first reducing the resident microbiota. In some laboratory experiments to date, amphibians first had their existing microbiota reduced by treatment with 3% hydrogen peroxide, antibiotics or both (Harris *et al.* 2009a; Becker & Harris 2010; Vredenburg *et al.* 2011) to open an accessible

Box 3 Threatened amphibian species in Madagascar

Madagascar, a global biodiversity hotspot, has over 400 species of amphibians, 99% of which are endemic (Fisher & Farrer 2011; Lötters *et al.* 2011). Much of Madagascar's rich amphibian fauna inhabits regions predicted by environmental niche modelling to be climatically suitable for *Bd* (Andreone *et al.* 2005; Rödder *et al.* 2009). Pond-breeding and stream-dwelling species are also predicted to be at risk of decline or extinction based on these life-history traits, and indeed, susceptibility trials indicate that tested Malagasy frog species will succumb to chytridiomycosis (C. Weldon, pers. comm.; Vredenburg *et al.* 2012).

Currently, *Bd* has not yet been documented on the island but its arrival is imminent (Lötters *et al.* 2011). Surveys of over 50 species from 12 localities of differing altitudes and biogeographical regions did not find *Bd* (Vredenburg *et al.* 2012). However, *Bd*'s dispersal ability is unquestionably high, considering its rapid spread around the globe, and therefore it is very likely to invade Madagascar. It has been proposed that human-mediated introduction of *Bd* via the amphibian trade is likely (Lötters *et al.* 2011). Once *Bd* is introduced, it has the potential to spread rapidly as seen in South America (Lips *et al.* 2008).

It is imperative to consider a prevention and mitigation strategy now in order to prevent catastrophic declines and extinctions in Madagascar like those seen in Central America and tropical Australia. Lötters *et al.* (2011) explain that effective responses for this potential threat include an increase in biosecurity, the development of breeding procedures for representatives of all major clades of Malagasy amphibians and the development of plans for 'emergency response'. We suggest that development of probiotic disease mitigation strategies should be included in conservation planning, as they will allow for a proactive response. Currently, nothing is known about the microbiota of Malagasy amphibians, and therefore the identification of anti-*Bd* bacteria is urgently needed. Ideally, broad-spectrum probiotics that are effective within certain frog assemblages or within particular habitats will be identified. Tropical montane regions are often optimal habitats for *Bd* growth, and in general probiotics should be developed for amphibians in habitats predicted to be at high risk for decimation by *Bd* (Fig. 3). Using the sampling strategies and filtering protocols developed here, probiotics can be identified and an extinction crisis can be averted.

niche for the probiotic (Reid *et al.* 2011). However, pre-treatment is not always necessary and could remove microbes that facilitate probiotic establishment or add defensive function. *Ps. reactans* was added successfully to *Pl. cinereus* without pre-treatment and led to lower morbidity effects in a laboratory experiment (Harris *et al.* 2009b). Similarly, in an agricultural study with probiotic treatment of wheat seeds, a probiotic's ability to increase yields in both laboratory and field trials was independent of pre-treatment disinfection (Pierson & Weller 1994). In the aquaculture literature, pre-treatment to reduce the existing microbiota typically is not done, and there have been many studies showing the efficacy of aquacultural probiotics (Verschuere *et al.* 2000; Kesarcodi-Watson *et al.* 2008). Applying a high density of the probiotic can be sufficient to ensure establishment, perhaps by giving the probiotic a competitive advantage. For individual treatment it also is necessary to determine the appropriate probiotic exposure time. In amphibian studies that showed a protective effect, a one-time probiotic bath between 2 and 48 h was used, suggesting that a bath within this time range is adequate for probiotic transmission (Harris *et al.* 2009a,b; Vredenburg *et al.* 2011).

Individual probiotic treatment has worked effectively in pond environments, where there is a high probability that pond-dwelling species can be captured (Vredenburg *et al.* 2011). In an aquacultural context, probiotic bath treatment of rainbow trout successfully reduced mortality (Gram *et al.* 1999). In agricultural contexts, seeds are often bacterised, which is analogous to probiotic bathing, and this treatment leads to improved survival (Haas & Défago 2005; Quagliotto *et al.* 2009).

In large-scale bioaugmentation field applications, hand-capturing amphibians and bathing frogs individually in probiotics is not possible in all situations, and environmental treatment may be a better option. When environmental treatment is feasible, it can be accomplished by soil or water inoculation. Studies in terrariums suggest that a probiotic can be established successfully in soil (Muletz *et al.* 2012). Suppressive soils, characterised by their ability to inhibit pathogens, have been added to agricultural environments to increase crop yield (Stutz *et al.* 1986), which suggests environmental inoculation is effective. The majority of amphibian species that have declined are aquatic breeders (Kriger & Hero 2007); therefore, inoculation of aquatic breeding sites could be a successful strategy. Studies to assess the efficacy of aquatic treatments are in progress. Environmental inoculation of aquacultural ponds can increase survival of farm-raised species (Moriarty 1998). For stream environments, it will be necessary for the probiotic to establish in the substrate such that bacterial reproduction is greater than emigration due to stream flow. Large-scale environmental inoculations are contingent on determining their efficacy and addressing safety concerns in small-scale field trials.

For both individual and environmental treatments, the optimal bacterial concentration to use and the optimal number of probiotic applications can be determined. For example, in aquacultural systems, probiotic concentration has been varied experimentally and effects on growth and survival have been assessed (Gatesoupe 1997). The time of appropriate within-year application for amphibians also needs to be determined and may be at the onset of breeding as treatment is likely to reduce pathogen transmission as well as increase probiotic transmission during mating aggregations. Many species with a larval stage experience mortality due to *Bd* spreading across the skin as keratinised epidermal tissues develops at metamorphosis; therefore, it is important to develop probiotics that are successful for larvae. Treating the larval stage can lower *Bd* transmission between larvae and post-larvae if both stages coexist in the same habitat.

Continued research is necessary to identify amphibian communities that are *Bd*-naïve so these communities can be prepared for *Bd* arrival. The optimal time of probiotic application in relation to *Bd* arrival also needs to be determined. In laboratory experiments, the probiotic control treatment (probiotic without *Bd*) has not caused any detectable morbidity or mortality (Harris *et al.* 2009a,b); therefore, areas in the path of an advancing *Bd* wave could be treated

before *Bd* arrives. It is also possible to treat amphibians as *Bd* begins to emerge. The recent probiotic field trial in the Sierra Nevadas was effective in treating mildly infected frogs (Vredenburg *et al.* 2011).

Species-specific individual treatment and community-based environmental treatment need not be mutually exclusive. The most desirable protocol may be to individually treat as many members of a population as possible and also to inoculate the environment with the same probiotic to establish or re-establish a self-disseminating system of defensive microbes. It is possible that the best probiotic for a highly susceptible species is not suited for other amphibian species in an assemblage or *vice versa*. Under these circumstances, the highly susceptible species could be treated individually with a specific probiotic bath, and the environment could be treated with a broad-spectrum probiotic intended for the amphibian community. This could reduce infection in less susceptible reservoir species, therefore contributing to protection of the highly susceptible species. In addition, environmental application could reduce transmission by killing zoospores in the environment.

ASSESSING EFFECTIVENESS

Ultimately, evaluation of success will be measured in terms of amphibian population survival and persistence. These measures can be estimated by visual encounter surveys and mark-recapture population size estimates (Heyer *et al.* 1994). Publication of all probiotic trial results is critical for effective protocol development and for avoiding repetition of unsuccessful experiments that are time-consuming and resource draining (Woodhams *et al.* 2012b).

BIOSAFETY

The addition of bacteria to an ecosystem has the potential to affect non-target species and ecosystem processes (Simberloff & Stiling 1996). Importantly, a probiotic should not negatively impact human health. Bacterially-produced compounds can be toxic to aquatic organisms. For example, violacein is acutely toxic to bacterivorous nanoflagellates (Matz *et al.* 2004), which may lead to increases in the bacterial community. Ecosystem processes such as decomposition or primary productivity also could be affected. It is not known whether the addition of anti-fungal bacterial species will have negative impacts on other microbial species; however, agricultural studies suggest that probiotic additions have minimal and transient effects on the microbial community structure (Scherwinski *et al.* 2008; Edel-Hermann *et al.* 2009). Nonetheless, precautions to minimise non-target effects and maintain the integrity of the ecosystem are essential.

CONTINUING RESEARCH AND FUTURE DIRECTIONS

Metabolite-based selection

An alternative probiotic selection approach is to begin with surveys of bacterially produced metabolites on amphibians in areas where populations are surviving with *Bd*. Currently, non-invasive, non-lethal screening techniques are being developed in collaboration with organic chemists that will allow researchers to detect and identify defensive metabolites on individuals. Bacteria that produce anti-*Bd* metabolites found in abundance on surviving amphibian hosts are likely to fulfil the most important effective probiotic criteria, that is, they inhibit *Bd in vivo* over a range of relevant environmental conditions and they persist on the host. In populations that are coexisting with *Bd*, common metabolites can be identified via HPLC-MS and linked to the bacteria that produce them either through known associations in the literature or through statistical methods that correlate metabolite presence with bacterial species' presence. The metabolites' defensive properties may be known or could be determined through *in vitro Bd* inhibition assays.

Probiotic mixtures

To date, most experimentation has used single-species probiotics; however, a mixture approach, where multiple bacterial species are used in synchrony, could be advantageous and should be explored (Gerritsen *et al.* 2011). In a field trial with wheat, some mixtures of fluorescent pseudomonads applied to seeds provided a significant increase in yield, whereas the use of individual strains was not effective (Pierson & Weller 1994). However, in a laboratory study of mussel larvae, a mixture of two probiotic strains did not improve survival over the effect provided by each strain individually (Kesarcodi-Watson *et al.* 2012). In one amphibian trial, a four-species probiotic mixture applied to infected *R. muscosa* did not persist on the host, but further research with other probiotic combinations is needed. Another approach is to transfer the microbial community from protected individuals to susceptible individuals as has been done successfully with faecal transplants in the human colon (Reid *et al.* 2011). A probiotic mixture could establish an anti-*Bd* community that works synergistically against *Bd* or include strains that inhibit pathogens through different modes of action. In addition, a mixture can allow treatment of multiple amphibian species and life-history stages that have different ideal probiotics in the same environmental inoculation.

Can probiotics offer a cure?

Research has concentrated on probiotics designed to prevent *Bd* infection. It is possible that probiotics will be able to cure or reduce established infections. Evidence suggests probiotic treatment can be effective if *Bd* infection is low at the time of treatment (Vredenburg *et al.* 2011). Additionally, highly infected individuals of *R. muscosa* have benefited temporarily from probiotic treatment in one study (Woodhams *et al.* 2012b). Treatment regimes involving conventional antifungal drugs and electrolyte treatments (Voyles *et al.* 2011; Woodhams *et al.* 2012b) followed by probiotic therapy can provide an additional way of treating established infections.

Probiotic strategies in other contexts

There are many papers in the agricultural and aquacultural literature that report improvements in growth, yield and survival from probiotic additions (Kesarcodi-Watson *et al.* 2008; Mohapatra *et al.* 2012). Protection from pathogens is one important function of probiotics (Berg 2009; Mohapatra *et al.* 2012). Selection of probiotics for disease mitigation typically involves *in vitro* inhibition trials with a pathogen and then application with baths, in feed, or by addition to the

environment for clinical trials. It is encouraging that both bathing of animals and seeds and environmental treatment as well as the use of single and multi-strain probiotics have been successful in agricultural and aquacultural contexts. These results suggest that the positive effects of probiotics can be independent of treatment protocols. In addition, several probiotic strains, such as some pseudomonads, appear to have a broad host range in plants and animals (Berg 2009), suggesting that community-based probiotics can be effective for amphibians. There is likely a bias against publishing negative results, which makes it difficult to arrive at general conclusions of what protocols to avoid; however, the wealth of documented success is encouraging.

The success of probiotics in agriculture, aquaculture and with amphibians suggests that such treatment could be extended to other wildlife groups. Protection afforded by probiotics may be helpful in repatriation efforts, such as those involving hellbender salamanders (*Cryptobranchus alleganiensis*). Repatriated individuals are raised from eggs in the laboratory and are likely to have atypical and depauperate microbial communities and naïve immune defences. Released animals are likely to be stressed, which increases disease susceptibility. Probiotic inoculation of hellbenders prior to repatriation is likely to improve their survival rate and is under investigation.

To date, there are no bioaugmentation studies on endangered groups or on wildlife species other than amphibians. However, other wildlife such as corals and bats are decimated by disease, and probiotics may be a plausible conservation solution, as suggested by Teplitski & Ritchie (2009) for corals. Bats in North America are threatened by the pathogen, *Geomyces destructans* (*Gd*), which causes white-nose syndrome (Gargas *et al.* 2009). It is conceivable that bats' skin bacteria can provide protection from *Gd* infection and might also work synergistically with host-produced defences (T. Cheng, pers. comm.). Since bats in Europe are able to survive *Gd* infection, it will be advantageous to screen these populations for protective microbes. *Gd* becomes pathogenic during hibernation; therefore, one goal should be to find probiotics that are inhibitory at the bat's hibernation body temperature and also during breeding when disease transmission is high. Our framework can help direct probiotic research to aid bat conservation.

CONCLUSION

Manipulation of microbial defences through the use of probiotics to alter disease outcomes in humans, agricultural and aquacultural species, and amphibians is a promising disease mitigation strategy. For amphibians, effective probiotics are integrated with other aspects of host immunity and offer the most feasible approach to date for combating the devastating effects of chytridiomycosis. Bioaugmentation of individual amphibians and of amphibian habitats with carefully selected locally occurring, anti-*Bd* microbes can be implemented in areas under imminent threat of *Bd* arrival, thereby mitigating the threat of chytridiomycosis in wild amphibian populations (Vredenburg *et al.* 2011). In addition, individuals in survival assurance colonies can be treated and successfully repatriated. We have outlined sampling strategies and filtering protocols that will guide conservation professionals in identifying the most promising probiotics. Wildlife is under increasing threat from fungal diseases (Fisher *et al.* 2012); therefore, continued optimisation of protocols is urgently needed so that these disease threats can be lessened through the use of probiotics.

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AUTHORSHIP

MCB composed the first complete draft with substantial input from AHL, SCB and RNH. DCW provided information on amphibian immunology. KPCM and AHL provided information on bacterial metabolites. MHB provided insights on the filtering process to select a probiotic. All authors contributed substantially to the revisions.

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