Using “omics” and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases


http://dx.doi.org/10.3389/fmicb.2016.00068

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Using “omics” and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases

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Running title: Using multi-omics for probiotic selection

Keywords: Probiotics, emerging diseases, metagenomics, transcriptomics, metabolomics, amphibians.

Number of words: 8,8199.095
Number of figures: 3
Number of boxes: 1
Using multi-omics for probiotic selection

Abstract
Emerging infectious diseases in wildlife are responsible for massive population declines. In amphibians, chytridiomycosis caused by Batrachochytrium dendrobatidis, Bd, has severely affected many amphibian populations and species around the world. One promising management strategy is probiotic bioaugmentation of antifungal bacteria on amphibian skin. In vivo experimental trials using bioaugmentation strategies have had mixed results, and therefore a more informed strategy is needed to select successful probiotic candidates. Metagenomic, transcriptomic, and metabolomic methods, colloquially called "omics", are approaches that can better inform probiotic selection and optimize selection protocols. The integration of multiple omic data using bioinformatic and statistical tools and in silico models that link bacterial community structure with bacterial defensive function can allow the identification of species involved in pathogen inhibition. We recommend using 16S rRNA gene amplicon sequencing and methods such as indicator species analysis, the K-S Measure, and co-occurrence networks to identify bacteria that are associated with pathogen resistance in field surveys and experimental trials. In addition to 16S amplicon sequencing, we recommend approaches that give insight into symbiotic function such as shotgun metagenomics, metatranscriptomics or metabolomics to maximize the probability of finding effective probiotic candidates, which can then be isolated in culture and tested in persistence and clinical trials. An effective mitigation strategy to ameliorate chytridiomycosis and other emerging infectious diseases is necessary; the advancement of omic methods and the integration of multiple omic data provide a promising avenue toward conservation of imperiled species.

1. Introduction
Emerging infectious diseases (EIDs) in wildlife pose a grave threat to biodiversity (Wake & Vredenburg, 2008; Blehert et al., 2009; Fisher et al., 2009; 2012; Price et al., 2014; Schrope, 2014). Examples of EIDs caused by fungal pathogens include white-nose syndrome in bats (Blehert et al., 2009) and chytridiomycosis in amphibians (Berger et al., 1998). The latter, caused by Batrachochytrium dendrobatidis (Bd), is considered the greatest disease threat to biodiversity at the current time (Wake & Vredenburg, 2008). Recently, a newly described chytrid fungal species, Batrachochytrium salamandrivorans (Bsal) has been identified as the causal agent of chytridiomycosis in salamanders and is causing many salamander populations declines in Europe (Martel et al., 2013; 2014; Yap et al., 2015). Several strategies have been proposed to contend against EIDs in amphibians (Fisher et al., 2012; Woodhams et al., 2012; McMahon et al., 2014; Langwig et al., 2015) including vaccination, selective breeding and the use of probiotic bioaugmentation (Harris et al., 2006; Woodhams et al., 2007; Harris et al., 2009; Stice & Briggs, 2010; McMahon et al., 2014; Hoyt et al., 2015). Successful implementation of these approaches for the conservation of wild populations will benefit from further laboratory and field-testing, particularly when informed by integrated multi-omics methods.

There is growing evidence that probiotic therapy in particular could be a promising approach to mitigating disease in a variety of organisms, including human, plant crops, and wildlife systems (Harris et al., 2009; Sánchez et al., 2013; Akhter et al., 2015; Forster & Lawley 2015; Hoyt et al., 2015; Papadimitriou et al., 2015). A relevant case comes from studies on plant-microbial interactions, which have identified several bacterial taxa involved in protection of plant crops against pathogens and extreme environmental stressor as well as in nutrient availability (Berlec 2012). Some of these microorganisms have been widely and successfully used as bio-fertilizers or biocontrols in plant agriculture (Bhardwaj et al., 2014; Lakshmanan et al., 2014). In amphibians, the approach that is currently being developed is probiotic bioaugmentation, which is the establishment and augmentation of protective bacteria-microbes that are already naturally occurring on at least some

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Using multi-omics for probiotic selection

individuals in a population or community (Bletz et al., 2013). Bioaugmentation has prevented morbidity and mortality otherwise caused by Bd during laboratory-based and field-based trials for some amphibian species (Harris et al., 2006; 2009). However, application of probiotics has been ineffective in other amphibian species (Becker et al., 2011; Küng et al., 2014; Becker et al., 2015a). The mixed success of probiotics could in part be caused by the selection of ineffective probiotic candidates because knowledge about the diversity of the microbiota and the ecological interactions occurring within these communities was lacking. For example, initial “training” of the immune system by early symbiotic colonists during development and priority effects of the microbial community, may exert strong influences on community resilience and colonization potential of probiotics (Reid et al., 2011; Hawkes & Keitt, 2015).

In an effort to improve the chances of a positive outcome from the use of amphibian probiotics, a protocol that filters out ineffective candidates has been proposed (Bletz et al., 2013). This method was designed to identify successful probiotics for disease mitigation and species survival based on culture-dependent data (Bletz et al., 2013). In addition to the Bletz et al. (2013) filtering protocol, a mucosome assay, which aims to measure the protective function of the skin mucus, has recently been developed and applied to test potential probiotics (Woodhams et al., 2014).

As new technologies and methods are being developed, it is desirable to further improve the filtering protocol with additional methods that can be used to facilitate probiotic candidate selection to increase the likelihood of success. In particular, high-throughput molecular techniques, colloquially called “omics” methods, have greatly increased our ability to characterize the taxonomic and genetic structure of bacterial communities, to estimate their functional capabilities and to evaluate their responses to stressors or pathogens (Grice & Segre, 2011; Fierer et al., 2012; Greenblum et al., 2012; Knief et al., 2012; Jorth et al., 2014). Some of the omics methods developed to date are gene amplicon sequencing, shotgun metagenomics, transcriptomics, proteomics and metabolomics.

Several studies and extensive reviews on these high-throughput molecular methods can be found in the literature (Fiehn 2002; Dettmer et al., 2007; Caporaso et al., 2011; Stewart et al., 2011; Altelaar et al., 2012; Caporaso et al., 2012; McGettigan 2013; Franzosa et al., 2014; Gust et al., 2014; Manor et al., 2014; Franzosa et al., 2015; Loman & Pallen 2015). Moreover, integrated multi-omics, which we define as the integrative analysis of data obtained from multiple omic methods, has the potential to greatly advance our understanding of ecological interactions occurring in microbial communities (Borenstein, 2012; McHardy et al., 2013; Meng et al., 2014). In this review, we establish an omics and integrated multi-omics framework with the aim of increasing the chances of selecting effective probiotic bacteria and achieving a successful disease mitigation strategy against EIDs. While these principles are applicable to other biological systems, for example in humans (Sánchez et al., 2013; Buffe et al., 2015; Forster & Lawley et al., 2015), we focus on applying these principles to the amphibian system, emphasizing currently accessible omics methods that have been explored in amphibians such as 16S rRNA gene amplicon sequencing (hereafter 16S amplicon sequencing), shotgun metagenomics, transcriptomics and metabolomics. However other omics methods such as proteomics could be relevant in future studies to understand the interactions between hosts, pathogens and host-associated microbial communities.

In this review, we will (1) provide relevant knowledge about the skin microbiome in amphibians; (2) proceed with a description of the omics and integrated multi-omics methods that have been or could be applied to the amphibian system; (3) describe how omics and integrated multi-omics approaches can be incorporated into a previously described filtering protocol to identify probiotic candidates (Bletz et al., 2013); (4) provide important considerations and future directions that are relevant to the
Using multi-omics for probiotic selection

success of probiotic selection supported by multi-omics data. It is important to note that the omics methods as well as the statistical, modeling and integrative methods mentioned in this review are only a subset of the current methods available and should therefore not be considered the only methods researchers can use to identify successful probiotic candidates.

2. Ecology of the amphibian skin microbiome

The amphibian skin microbiome is defined as the microbiota and its combined genetic material present on the skin. Determining the main drivers of the assembly of the skin microbiome through the use of omic methods and culture-dependent approaches may greatly enhance our ability to develop successful probiotic treatments and prevent amphibian population declines caused by chytridiomycosis.

The amphibian skin microbiome is determined by the microbiota’s interactions with host-associated factors and with abiotic and biotic factors (Figure 1, Box 1). Host-associated factors include host genetic diversity and the adaptive and innate immune systems (Box 1A), in addition to host behavior, ecology and development. Biotic factors include ecological interactions between skin symbiotic microbes and the microbial composition of environmental reservoirs (Box 1B), and abiotic factors include environmental conditions such as temperature and humidity (Box 1C). Altogether, the factors that influence the skin microbiome of amphibians determine the chemical composition of the skin mucus and in turn help determine the degree of host susceptibility against pathogens (Searle et al., 2011; Woodhams et al., 2014). Box 1 summarizes the current state of knowledge on the drivers of the amphibian skin microbiome. We now focus on omics and integrative multi-omics methods and how they can be used to address knowledge gaps that are key to developing effective probiotic strategies.

3. Omics methods to identify probiotic candidates

An important first step toward the identification of potential probiotics in amphibians is to determine differences in the structure and function of skin microbial communities in the presence or absence of Bd. This approach includes comparing diseased and not diseased individuals after exposure to Bd in laboratory trials, as well as examining Bd-tolerant or resistant species from localities that have experienced population declines. The assumption is that individuals or species that persist in the presence of Bd might harbor protective bacteria that allowed them to survive. Such studies can be done through 16S amplicon sequencing of the whole microbial community (Caporaso et al., 2012). Furthermore, in order to identify key bacterial species responsible for pathogen protection it will be necessary to go beyond taxonomic descriptions and determine the functional capacities of the community through the use of additional techniques such as shotgun metagenomics, metatranscriptomics and metabolomics. These approaches can be used to identify bacterial strains that contain genes whose function could make them an effective probiotic. For example, based on previous knowledge about bacterial interactions, searching for genes associated with the production of antibiotics (including anti-fungal metabolites) and beneficial host-microbe interactions could increase the chances of selecting good probiotic candidates.

It is important to emphasize that the use of omic approaches to identify probiotics will only be relevant if these are linked with biological assays of culturable bacteria (sensu Becker et al., 2015b). Linking culture-independent with culture-dependent data is a fundamental step towards the identification of successful probiotics (Walke et al., 2015). Importantly, if bacterial cultures with inhibitory activities are available, then physiological, metabolic and genomic analyses of these strains can greatly inform omics predictions. Below, we describe currently available omic approaches and their applications for probiotic selection in amphibians.

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3.1. 16S amplicon sequencing

Amplicon sequencing is the sequencing of a particular gene or gene fragment of an entire microbial community through the use of high-throughput sequencing methods (Metzker 2010). In particular, 16S amplicon sequencing of skin bacterial communities has allowed us to determine the most prevalent and relatively abundant bacterial OTUs (operational taxonomic units) on different amphibian species and populations, and across life-history stages (McKenzie et al., 2012, Kueneman et al., 2014, Loudon et al., 2014a, Walke et al., 2014). By providing information about which bacterial taxa appear to be involved in pathogen protection, 16S amplicon sequencing can help target the isolation of potential probiotic bacteria in pure culture. This can be accomplished using data from both field surveys and laboratory experiments.

Field surveys of amphibians naturally-exposed to Bd can allow the tracking of changes in the microbial community structure in response to Bd infection. For example, recent studies in Rana sierrae populations have shown a clear correlation between specific OTUs and Bd infection intensity in a field survey (Jani & Briggs, 2014). Field studies can therefore inform us about the bacterial taxa that increase in abundance in the presence of Bd and might therefore be involved in a concerted response to the infection (Rebollar et al., 2015). This is an essential step to direct the isolation of potential probiotic bacteria in order to test their ability to inhibit Bd.

Field surveys, however, are inadequate to determine causal relationships between OTU presence and pathogen presence. Experimental laboratory Bd exposures are essential to determine changes in the microbial structure in response to Bd infection so they can provide information about which bacterial taxa could be involved in host defense against pathogens. Variation in susceptibility to Bd has been linked to changes in cutaneous bacterial community structure (Becker et al. 2015a, Holden et al., 2015), presence of skin antifungal metabolites (Brucker et al., 2008; Becker et al. 2009; Becker et al. 2015b), function of the mucus components (Woodhams et al., 2014), and MHC genotype (Savage & Zamudio, 2011, Bataille et al., 2015). For example, an experiment investigating the use of probiotics to prevent chytridiomycosis in the highly susceptible Panamanian golden frog (Atelopus zeteki) demonstrated that individuals that were able to clear Bd infection harbored a unique community of bacteria on their skin prior to probiotic treatment (Becker et al., 2015a). Furthermore, the authors identified several bacterial families on surviving frogs that were correlated with clearance of Bd (Flavobacteriaceae, Sphingobacteriaceae, Comamonadaceae and Rhodocyclaceae). In contrast OTUs on individuals that died belonged to the families Micrococccineae, Rhizobiaceae, Rhodobacteraceae, Sphingomonadaceae and Moraxellaceae (Becker et al., 2015a). To determine if the OTUs associated with survival could prevent chytridiomycosis in A. zeteki, the next steps would be to isolate the potentially beneficial OTUs from surviving golden frogs, test the isolates for Bd inhibition in vitro (Bell et al., 2013) and/or using mucosome assays (Woodhams et al., 2014), and finally test the resistance of inoculated individuals to Bd infection (Bletz et al., 2013). In addition, the ability to predict host susceptibility via 16S amplicon sequencing may provide a useful tool for captive population managers to identify individuals that could be used for reintroduction trials.

A number of studies using 16S amplicon sequencing have detected bacterial community members that persist independent of distinct environmental reservoirs (Loudon et al., 2014a), time in captivity (Becker et al., 2015a), and in different developmental stages (Kueneman et al., 2014). These prevalent and persistent community members may be closely associated with their hosts over evolutionary timescales. If important for disease defense, OTUs identified in these studies may also provide probiotic candidates that are effective at persisting on hosts, and even naturally transmitted between hosts or across generations (Walke et al. 2011). Augmenting these bacteria in the habitat
Using multi-omics for probiotic selection

may also provide disease mitigation benefits (Muletz et al., 2012). Moreover, amplicon sequencing is not limited to bacterial identification but it can also unravel the diversity of micro-eukaryotes through the sequencing of the 18S rRNA gene. For instance, a recent study characterized the bacterial and fungal composition of amphibian skin communities, and determined changes in fungal diversity across different developmental stages (Kueneman et al., 2015).

3.2. Shotgun metagenomics

The sequencing of the total microbial community DNA known as shotgun metagenomics, has provided information about the genes present in microbial ecosystems (Barberán et al., 2012b; Knief et al., 2012; Xu et al., 2014). Metagenomic information can allow the identification of genes or genetic pathways associated with specific functions, and therefore it can provide useful information about the potential functional capabilities of microbial communities. For example, metagenomic approaches in marine symbiotic systems have revealed some of the capabilities of bacterial symbionts that are important for interaction with their hosts such as genes involved in nutrient availability and recycling of the host’s waste products (Woyke et al., 2006; Grzymski et al., 2008). As mentioned previously, in vitro inhibition assays with bacterial isolates cultured from amphibian skin have detected many bacterial strains with antifungal activities (Harris et al., 2006; Holden et al., 2015; Woodhams et al., 2015). Using metagenomics, these antifungal activities, such as the ability to produce extracellular secondary metabolites, can be identified and bacterial species containing these genes could be inferred. However, metagenomic inferences rely on how much information is available in databases and how much we know about antifungal genetic pathways of isolates in culture. Nonetheless, the comparison of shotgun metagenomic data from resistant and/or tolerant frogs will be very helpful for identifying potential bacterial candidates for probiotics. Bacteria whose genomes contain antifungal gene pathways and pathways associated with the ability to colonize and persist can be identified, which can narrow down the number of probiotic candidates.

3.3. Metatranscriptomics

Metatranscriptomics is the analysis of the mRNA expression profiles in a community and is relevant for identifying genes or genetic pathways that are up or down regulated in response to a pathogen infection. This method could also unravel functional responses involved in bacterial-host interactions such as the expression of adhesion genes or additional traits associated with bacterial colonization and attachment to eukaryotic hosts (Klemm & Shenbri, 2000; Dale & Moran, 2006; Kline et al., 2009; Chagnot et al., 2013). Determining the capacity of different bacteria to colonize the skin of the host is extremely relevant for selecting bacterial probiotic candidates. A metatranscriptome approach has shown differences in gene expression in human oral microbiomes between healthy and diseased individuals, and specific metabolic pathways associated with periodontal disease have been identified (Jorth et al., 2014). Metatranscriptomes of fungi and algae in symbiosis with plants and corals, respectively, have also revealed changes in gene expression in response to stressors and environmental cues (Gust et al., 2014; Liao et al., 2014).

Metatranscriptomics may be a good approach in experimental laboratory settings, in which amphibians are exposed to Bd. This will allow for the identification of genes that change expression levels in response to pathogen infection and could be associated with host survival. To our knowledge, no studies have used a metatranscriptome approach to study the amphibian skin microbiome, in part because acquiring enough bacterial mRNA from amphibian skin is difficult. To pursue a metatranscriptome approach in amphibians it will be important to improve sampling strategies and molecular methods that increase the bacterial mRNA yield and reduce the proportion

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Using multi-omics for probiotic selection

of eukaryotic mRNA from the host and from fungi present on the skin (Stewart et al., 2011; Giannoukos et al., 2012; Jorth et al., 2014).

An additional research avenue would be to conduct transcriptomic studies on amphibian hosts (Ellison et al., 2014, Savage et al., 2014; Price et al., 2015) and in parallel measure changes in the skin microbiome (using 16S amplicon sequencing or metatranscriptomics) in the context of disease or probiotic application. Understanding the role of the genes expressed in host immune responses in shaping the microbiota that colonize and persist on the host may allow novel insights for disease treatment (Box 1A). Moreover recent methods like dual RNA-seq, which aim to determine the expression profiles of both the host, and the associated microbiota (including pathogens), may allow to determine the interactions occurring between skin microbiota, the pathogen and the host (Westerman et al., 2012; Schulze et al., 2015). These interactions may provide useful insights for understanding infection dynamics and informing probiotic design.

3.4. Metabolomics

Metabolites are the chemical intermediates and final products of cellular processes, and system-wide attempts to document all chemical species present in a selected biological sample (i.e., metabolomics) have been undertaken for over a decade (Fiehn 2002, Bouslimani et al., 2015). In amphibians, differences in skin metabolite profiles (representing the sum of host and microbiologically-produced metabolites) across species have been identified (Umile et al., 2014). Metabolomics could also be used to compare species, populations or individuals with varying susceptibility to pathogens like Bd. For example, metabolite profiles can be compared between naïve populations and populations that have survived an epidemic of Bd, and metabolites that appear among survivors can be identified. The bacteria that produce these metabolites can then be tested for their inhibitory properties and probiotic potential.

A complementary approach is to experimentally expose amphibians to Bd and compare the metabolite profiles of survivors and non-survivors. Individuals of the salamander Plethodon cinereus that were exposed to Bd and survived had significantly higher concentrations of the metabolite violacein on their skins than did individuals that died (Becker et al., 2009). This metabolite is produced by several species of bacteria, most notably Janthinobacterium lividum, which lives on the skin of many amphibian species and to inhibit Bd in vitro (Harris et al., 2006). Moreover, use of J. lividum as a probiotic on Rana muscosa decreased morbidity when individuals were exposed to Bd (Harris et al., 2009), although extension to another host species, the Panamanian golden frog (A. zeteki), failed to provide similar protection (Becker et al., 2011).

One challenge in metabolomics is that metabolites vary enormously in chemical structure and reactivity, making the use of a single analytical tool to create a “chemical master inventory” nearly impossible. High-resolution mass spectrometry, often coupled with a separation technique such as high-performance liquid chromatography (LCMS), has led to significant strides in this arena (Dettmer et al., 2007). Since LCMS does not automatically provide molecular structure, further analysis is required, which can involve comparison to molecular databases. Free-access compendia of metabolite data have been published as early as 2005, in the first metabolomics web database METLIN, as well as the subsequent Human Metabolome Database (HMDB; Wishart et al., 2007; 2009; 2013). Another challenge arises from the sheer number of data points generated by such analyses, the visualization of which can be daunting, although multivariate statistical analyses and analytical methods have been presented to address this chemometric challenge (Sharaf et al., 1986; Patti et al., 2013; Bouslimani et al., 2015).
4. Statistical tools to identify probiotic candidates and integrate multi-omics data.

4.1. Identifying key bacterial species associated with amphibian survival against *Bd*

There are several statistical tools that can be used to identify OTUs that are driving differences at the community level between two or more groups (e.g., susceptible and non-susceptible individuals). For example, indicator species analysis (Dufréne & Legendre, 1997) provides a method to identify indicator OTUs based on the relative abundance and relative frequency of each OTU in predefined groups. In this analysis, each OTU is given an indicator value ranging from one to zero. An OTU that is observed in all the frogs of one group and absent from the other would be designated an indicator value of one. In contrast, an OTU that is equally distributed across both groups would have an indicator value of 0. Statistical significance of each value is then calculated with Monte Carlo simulations. Indicator species analysis can be performed with the IndVal function in the *labdsv* package (Roberts, 2007) of the R statistical software (R Core Team, 2014).

An additional statistical technique is the K-S Measure (Loftus et al., 2015), which is an extension of the Kolmogorov-Smirnov (K-S) test statistic (Kolmogorov 1933, Smirnov 1936). While the K-S test statistic has long been used to assess differences in empirical distribution functions between two groups, the K-S Measure was designed to assess differences in the distributions of the relative abundances of individual OTUs among K > 2 groups. For a given OTU, empirical relative abundance distribution functions are assembled for each group using the data for all individuals assigned to that group. The K-S Measure simultaneously assesses the magnitude of the differences between the distributions, using the weighted sum of the K-S statistics for all pairwise comparisons of distributions defined by K groups. The K-S Measure ranges from zero to one, where values closer to one imply greater differences between the K distributions than values closer to zero (Loftus et al., 2015).

The linear discriminant analysis (LDA) effect size method, LEiSe, can also be an informative method (Segata et al., 2011). LEiSe can be used to compare among groups that are biologically relevant and determine which features (organisms, clades, OTUs, genes, or functions) are significantly different (Albanese et al., 2015, Clemente et al., 2015, Zeng et al., 2015). LEiSe determines the factors that most likely explain differences between classes by coupling standard tests for statistical significance (Kruskal Wallis and Wilcoxon non-parametric tests) with additional discriminant tests that estimate the magnitude of the effect (LDA score).

Another promising analysis technique is DESeq2, which offers higher power detection for smaller sample sizes (less than 20 samples per group) compared to traditional non-parametric tests based on Kruskal-Wallis and Wilcoxon rank-sum approaches (McMurdie & Holmes, 2014; Weiss et al., 2015). While the non-parametric tests do not assume a distribution, DESeq2 assumes a negative binomial distribution to obtain maximum likelihood estimates for a feature’s (gene, OTU, etc.) log-fold change between two groups (Anders & Huber, 2010; Love et al. 2013). Bayesian shrinkage is then used to reduce the log-fold change toward zero for those OTUs of lower mean count and/or with higher dispersion in their count distribution. These shrunken log-fold changes are tested for significance with a Wald test. If the average number of sequences per sample between the two sample groups differs greatly (>3x), it is better to use a Kruskal-Wallis type approach such as LEiSe for lower type 1 error. All methods, indicator species, K-S Measure, LEiSe and DESeq2, take into account the relative abundance and prevalence of each OTU with the latter two methods allowing for a stratified statistical design with biologically relevant classes and subclasses.

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We suggest that all or some of these statistical methods can be used in parallel to identify taxa involved in protection against pathogens. Importantly, some of these statistical tools can be used to identify OTUs based on 16S amplicon sequencing but they can also be used to identify genes and metabolites associated with pathogen protection based on metagenomics, metatranscriptomics and metabolomics data (Segata et al., 2011, Love et al., 2013; Loftus et al., 2015; Weiss et al., 2015).

### 4.2. Defining interactions and networks involved in protection against pathogens

One inherent challenge of omic data is interpreting the complex interactions present within the data collected. Many microbial datasets can have more than 5,000 features (e.g., OTUs in the case of 16S amplicon sequencing), so this implies almost 12.5 million possible two-feature correlations. Also, it is expected that within these complex microbial communities three or more feature interactions will occur. Furthermore, omic datasets exhibit diverse challenges, including only providing relative abundances based on a fixed total number of sequences rather than absolute abundances, or the abundance and spacial distribution of zeroes in a data matrix (compositionality) (Aitchison, 1986; Lovell, 2010; Friedman & Alm, 2012). Data sets with many zeroes, missing data due to incomplete sampling, and ecological relationship diversity (e.g. parasitism, commensalism, etc.) further complicates statistical analysis (Reshef et al. 2011; Friedman & Alm, 2012). However, despite the challenges, computation is possible in terms of time and expense as compared with evaluating more than 12.5 million microbial interactions in the laboratory. Also, the mathematical and statistical approaches for analyzing community data are improving. One technique for inferring microbial interactions from sequencing data is correlation network analysis. Networks consist of “nodes” (OTUs, genes, metabolites, integrated omics) and “edges”, based on the strength of the interaction between nodes, and which imply a biologically or biochemically meaningful relationship between features (Imangaliyev et al., 2015). Interaction values between nodes are commonly referred to as co-occurrence patterns (Faust & Raes, 2012).

Many different techniques have been developed for assessing correlations and constructing interaction networks. Some classic correlation techniques are the Pearson correlation coefficient (Pearson 1909), which assess linear relationships, or the Spearman correlation coefficient (Spearman 1904), which measures ranked relationships. Both Pearson and Spearman correlation are very useful (e.g. Arumugam et al., 2011; Barberan et al., 2012a; Buffie et al., 2015), however neither was developed specifically for the challenges of sequencing data, e.g. compositionality. Of the two, Spearman is less adversely affected by the former challenges. Other correlation methods that have been developed include CoNet (Faust et al., 2012), MENA, or Molecular Ecological Network Analysis (Zhou et al., 2011; Deng et al., 2012), Maximal Information Coefficient (MIC) (Reshef et al., 2011), Local Similarity Analysis (LSA) (Beman et al., 2011, Ruan et al., 2006, Steele et al., 2011, Xia et al., 2013), and Sparse Correlations for Compositional Data (SparCC) (Friedman & Alm, 2012). Network visualizations are often performed in the igraph package in R (R Core Team, 2014) or in Cytoscape (Shannon et al., 2003).

For probiotic selection, the construction and analysis of networks can infer which taxa occur together in natural communities, and can attempt to identify the direction of interactions between taxa or groups of highly connected taxa (Barberán et al., 2012a). For example, correlation networks in human and mouse models helped identify *Clostridium scindens* as exhibiting a negative correlation pattern with the pathogen *Clostridium difficile*. Transfer of *C. scindens*, either alone or with other bacteria identified by the correlation networks, was then experimentally shown to increase resistance to *C. difficile* infection in mouse models (Buffie et al., 2015). In the case of amphibians, networks
Using multi-omics for probiotic selection

that integrate bacterial and fungal omics data taken from hosts, can inform our understanding of interactions occurring between diverse bacterial and fungal taxa (Figure 2A). Determining the negative or positive correlations that shift in the presence of a pathogen like Bd in experimental trials could help distinguish groups of microbes (mainly bacteria and fungi) involved in resistance against pathogens (Figure 2B).

In order to identify potential probiotics against Bd in amphibians, correlation networks can be used to compare individual or group interactions in omic data between (1) Bd-positive and Bd-negative populations in the field, (2) Bd-infected and uninfected hosts in experimental trials, (3) hosts with differential Bd infection intensity in the field or in experiments and (4) hosts from different life stages. However, caution is warranted when inferring a mechanism of interaction based solely on patterns of correlation (Levy & Borenstein, 2013). Targeted culturing of taxa identified by networks may additionally be used to inform probiotic selection and test their ability to inhibit Bd singly or jointly (see Section 5.2), as there may be synergistic Bd inhibition (Loudon et al., 2014b). These taxa can be the basis for forming specific hypotheses that can be explored in experimental studies such as a probiotic treatment to determine if the addition of these species to amphibian skin can establish, persist, and increase the anti-Bd function of the microbial community of susceptible species.

4.3. Integrating multi-omics data to identify anti-fungal genetic or metabolic pathways

Metagenomics, metatranscriptomics and metabolomics are important tools to determine molecular pathways present in microbial ecosystems. One of the main goals is integrating these multiple massive data sets to distinguish community patterns associated with a specific function such as host disease resistance. Protection against Bd in amphibians is likely achieved by a combination of functional pathways present in the skin microbiome in concert with the host’s immune system. Therefore, the integration of multiple high dimensional datasets using predictive computational approaches such as bioinformatic predictive tools, multi-omic correlations and in silico models are key to predict functional outcomes within the skin microbiome (Borenstein, 2012; Langille et al., 2013; McHardy et al., 2013; Meng et al., 2014). One approach termed Reverse Ecology, offers a promising way to use high-throughput genomic data to infer ecological interactions from complex biological systems (Levy and Borenstein, 2012; 2014). It involves predicting the metabolic capacity of a biological system (including symbiotic systems) based on metagenomic data through the use of graph-theory based algorithms and genome-scale metabolic networks (Borenstein et al., 2008; Borenstein & Feldman, 2009; Freilich et al., 2009; Levy & Borenstein, 2012, Moran et al., 2014). To date, the amphibian skin microbiome has mainly been described using culture-dependent techniques and 16S amplicon sequencing. The use of these additional techniques may greatly improve our understanding of this microbial system and could allow us to identify fundamental metabolic pathways and ecological networks associated with defense against pathogens like Bd. For example, multi-omic correlations of 16S amplicon sequencing and metabolomics (McHardy et al., 2013) may allow us to determine bacterial taxa and metabolites associated with Bd inhibition in Bd-tolerant species and in individuals exposed to Bd in experimental trials.

Moreover, the bacterial taxa that produce the metabolites could be determined by statistical methods that associate metabolite presence with bacterial species’ presence. For example, using random forest with machine learning one can rank microbes by relative contribution (importance) (Knights et al., 2011a; 2011b; 2011c; Ditzler et al. 2014). Random forest is an accurate machine-learning multi-category classification algorithm for linking abundances of microbial taxa to physiological states such as metabolite production or immune function (Statnikov et al. 2013). Bacterial species that

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produce one or more anti-

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Bd metabolites that are associated with survival in a Bd-positive

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environment would be excellent probiotic candidates for bioaugmentation in at-risk populations.

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5. Using omics and integrating multi-omics data to inform probiotic selection through a

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filtering protocol

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Bletz et al. (2013) recently outlined sampling strategies and screening protocols for identifying ideal

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probiotics for amphibians (Figure 3). The framework involves (1) collecting and culturing skin

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microbes from selected host species; (2) isolating all morphologically-distinct colonies into pure

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culture; (3) testing each isolate for its ability to inhibit Bd in vitro (Bell et al., 2013); (4) testing

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highly inhibitory isolates for their ability to colonize and persist on amphibian skin; (5) and for those

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isolates that persist testing their ability to protect the host against Bd infection in clinical trials in the

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laboratory, followed by field trials (Bletz et al., 2013). In the case of the skin of some amphibian

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species, the dominant members of the microbiota are readily cultured (Walke et al. 2015), whereas

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some rare but prevalent members identified by 16S amplicon sequencing have been difficult to

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isolate in culture (Loudon et al. 2014a). Specialized media may be necessary to target microbes

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identified by omics approaches including not only bacteria but also fungi. Even though most of the

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probiotic search in amphibians has focused on bacterial candidates, the filtering protocol proposed by

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Bletz et al. (2013) could be also used to target potential fungal probiotics. Omic datasets and the

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integration of multi-omic analyses can facilitate the selection of the probiotic candidates that progress

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through this sampling and screening protocol. Below we describe the steps of the filtering protocol

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(Bletz et al., 2013) and the mucosome assay (Woodhams et al., 2014) that can be improved by omics

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and the integration of multi-omic approaches (Figure 3).

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5.1. Using omics data to inform the isolation of probiotic candidates.

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A probiotic approach typically requires culturing and isolation of microbial species in order to test

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their antifungal functions and use only those species with desired properties. Given the taxonomic

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diversity of the bacterial component of the amphibian skin microbiome (Kueneman et al., 2014;

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Loudon et al., 2014a, Walke et al., 2014; Becker et al., 2015a, Kueneman et al., 2015), it would be

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useful to reduce the number of bacteria that one is trying to isolate and test for

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inhibition. Omics methods can streamline the bacterial isolation process by identifying promising

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probiotic taxa, which can then be isolated using media and culture conditions that favor or enrich for

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specific bacterial groups (Watte et al., 2000; Connan & Giovannoni, 2002; Rappé et al.,

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2002; Zengler et al., 2002, Vartoukian et al., 2010).

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Through the integration of multi-omics data, bacterial community members that are

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associated with surviving amphibian populations in the field and in laboratory experiments can be

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identified. We suggest using several methods such as indicator species analysis, the K-S Measure,

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LEfSe and co-occurrence networks in parallel to identify probiotic candidates. The main goal of

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using several methods is to obtain a list of OTUs that are congruent among methods. OTUs

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suggested as probiotic candidates by these culture-independent methods can be matched to bacterial

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isolates in pure culture identified with 16S rRNA Sanger sequencing (Woodhams et al., 2015) or to

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bacterial strains whose whole genome has been sequenced. These isolates would then proceed to

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testing for inhibition against Bd using in vitro challenge assays (Bell et al., 2013) or mucosome

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assays (Woodhams et al., 2014).
Using multi-omics for probiotic selection

5.2. Using omics to predict if probiotic candidates should be tested individually or in combination.

Data obtained by omics methods can be useful for determining if single species or combinations of species are optimal for probiotic inoculation. Isolates can be chosen based on co-occurrence networks and genetic or metabolic pathways enriched in hosts that survived in the presence of Bd or that cleared Bd infections.

Single isolate probiotics have been successful in some systems such as the probiotic bacterium J. lividum in experimental trials with the host R. muscosa experimental trials (Harris et al., 2009). However, research in several symbiotic systems has shown that bacterial mixtures are necessary to exert a protective effect against pathogens and a restorative effect on hosts (Lawley et al., 2012; Fraune et al., 2014). For example, in a mouse model of C. difficile infection, a six-species probiotic mixture led to a community reset and recovery from C. difficile infection (Lawley et al., 2012). The authors speculated that the six species in combination were successful due to their phylogenetic distinctiveness, which allowed them to more effectively fill available niche space. Importantly each species alone was not curative, but each species was necessary in the mixture for the treatment to be effective (Lawley et al., 2012).

We currently do not know when a one-species probiotic or when a mixture will be more effective against Bd in amphibians. Omics data might offer insight into why single probiotics have failed in some cases. In addition, the integration of multi-omic data could be used to choose sets of isolates that might work in concert based on the presence of facilitative interactions among them from co-occurrence networks or based on the existence of complementary components of genetic or metabolic pathways.

5.3. Using omics data to track the effectiveness of probiotic bacteria in laboratory and field trials.

Omics can be used to determine if a probiotic was able to colonize, persist and/or trigger antifungal pathways in the symbiotic community. This is key to its success as a probiotic (Bletz et al., 2013). We hypothesize that this can be accomplished if the community reaches an alternative stable state once the candidate taxon is applied and community structure begins to shift in response (Faust & Raes, 2012; Fierer et al., 2012). The new stable state of the community must have antifungal functions and sufficient competitive abilities against invading pathogens to protect the host. Co-occurrence networks could be helpful to track whether bacterial interactions remain stable or shift through time after probiotic application (Rosvall & Bergstrom, 2010). In addition, these approaches could be useful for understanding whether probiotics applied during one life stage persist and remain effective in subsequent life stages (e.g., through metamorphosis).

One of the ultimate goals of probiotic bioaugmentation is for it to be used to reintroduce Bd-susceptible amphibian species back into their natural habitats. Thus, candidate probiotics must persist on the host and function appropriately not only in laboratory settings, but also in the host organism’s natural environment. In addition, an ideal probiotic would not disturb other microbial systems, including those of non-target host organisms, upon introduction. This is particularly important to consider if the planned mode of delivery or maintenance of the probiotic is via the soil or water (Muletz et al., 2012). Similar to laboratory trials, it will be important to collect and evaluate “before and after” metagenomic, metatranscriptomic and metabolomic data to better understand the responses.
Using multi-omics for probiotic selection

of both the host organism and microbial systems in the surrounding environment to probiotic
application.

5.4. Using omics approaches to inform probiotic testing in mucosome assays

The integrated defenses of the amphibian skin mucus, including antimicrobial peptides, microbiota,
mucosal antibodies, lysozymes and alkaloid secretions, are called the mucosome. A mucosome assay
developed by Woodhams et al. (2014) can be used to predict the infection prevalence of Bd-exposed
populations and the survival outcome upon exposure. Briefly, a mucosome assay consists of placing
individuals in a bath that collects their mucosal secretions. The secretions are used for in vitro
viability assays, in which they are tested for their ability to kill Bd. This assay accurately predicts
adult amphibian survival upon Bd exposure (Woodhams et al., 2014).

Importantly, the mucosome assay can be used to measure and predict the effectiveness of probiotic
treatments. Probiotic candidates identified by omics analyses need to be isolated through culturing
methods and then added to amphibian skins to evaluate their effectiveness using a mucosome assay.
This is accomplished by comparing the mucosome function before and after the addition of a
probiotic bacterium or a group of probiotic bacteria. Probiotic candidates that pass the preliminary
assay screen would then be ready for persistence and clinical trials (Figure 3D). The advantage of
using the mucosome assay is that it could minimize the need to expose amphibians to Bd in clinical
trials, which is particularly relevant in the case of endangered species or species that are naïve to the
disease.

6. Important considerations and future directions

To facilitate the identification of successful probiotic candidates, we recommend using an
interdisciplinary approach. The interaction and collaboration of scientists who have different
expertise as well as interaction with natural resource managers can greatly improve the outcomes of
probiotic research.

In addition to probiotic therapy for the reintroduction of species currently being held in captivity, one
important challenge is to identify probiotic candidates for species that are still naïve to pathogen
infections. Two relevant cases from highly diverse regions are frogs in regions of Madagascar that
have not been exposed to Bd (Bletz et al., 2015) and North American salamanders that are so far
naïve to Bsal (Martel et al., 2013; 2014; Yap et al., 2015).

Omic methods, along with mucosome assays and culture-dependent methods, may greatly improve
our knowledge on the capacity of naïve individual amphibians (and their microbio) to contend
against novel pathogenic infections. In addition, other non-omic techniques such as real-time PCR
(qPCR), fluorescence in situ hybridization (FISH) and mass spectrometry of culturable communities
may greatly inform probiotic discovery since they can increase our understanding of the amphibian
skin microbiome dynamics (Watrous et al., 2012; Barea et al., 2015). This is a fundamental step for
developing effective probiotics that could be applied to at-risk amphibian populations.

In addition to the previous research on the amphibian system, this review has mainly focused on
bacterial probiotics. However, future research may benefit by considering the micro-eukaryotic and
viral components of the skin community. Indeed, fungi are important components of mammalian and
amphibian skin (Underhill et al., 2014; Kueneman et al., 2015), and viruses have been linked to
dysbiosis in the oral cavity (Edlund et al., 2015). Several studies have examined the importance of
Using multi-omics for probiotic selection

non-bacterial microbiota in host health (Parfrey et al. 2014; Rizzetto et al., 2015). Indeed, the co-
occurrence of diverse microbiota can drive conflicting immune responses and cause trade-offs (Susi
et al., 2015).

In addition to altering the microbiota with probiotic bioaugmentation applications, prebiotics, which
are non-digestible carbohydrates, may also have beneficial effects (Patel & Denning, 2013).

Prebiotics can alter the nutrient sources to selectively favor targeted microbes as in the case of the
prebiotics applied in aquaculture systems (Ringø et al., 2010; Akhter et al., 2015). This line of
research has only being applied in the intestinal system (Gourbeyre et al., 2011, Patel & Denning,
2013). Thus further research is needed to determine how prebiotics and the combination of probiotics
and prebiotics (synbiotics) could be applied to the amphibian system to favor the colonization and
growth of antifungal microbes. Moreover, the use of bacterial metabolic products from probiotic
microorganisms (postbiotics, Patel & Denning, 2013) or the addition of non-replicating probiotics
(postbiotics; Patel & Denning, 2013) might also be a promising research avenues toward mitigation
of emerging infectious diseases.

7. Conclusions

Omic methods provide us with the opportunity to thoroughly describe microbial symbiont
communities and to determine their structure and functionality. In particular, the skin microbiome in
amphibians can be elucidated through the integration of multi-omics data to identify potential key
beneficial microbiota and the antifungal genetic and metabolic pathways involved in protection
against Bd or Bsul. Disease mitigation through bioaugmentation can be and has been applied to other
biological systems, such as bats fighting against white nose syndrome disease, as well as in cattle
raising, agriculture, and aquaculture systems (Kesarodci-Watson et al., 2008; Ringø et al., 2010;
Bhardwaj et al., 2014; Lakshmanan et al., 2014; Hoyt et al., 2015; Papadimitriou et al., 2015; Uyeno et
al., 2015). These systems share similar concerns and also have the difficulties that we have described
here in finding mitigation solutions, so they could benefit from this omics approach. We have a clear
framework for selecting an ideal probiotic (Bletz et al., 2013); however, integrative multi-omics can
prioritize candidates and facilitate selection of candidates to move to the next steps in the filtering
protocol. Finding effective probiotics has the potential to reduce the large losses of biodiversity from
emerging infectious diseases such as chytridiomycosis.

Box 1. Factors influencing the amphibian skin microbiome

A. Host-associated factors: genetic and immune system diversity

Due to the essential function of amphibian skin in protection of the host against desiccation and
pathogens, the skin mucus is a niche with unique chemical properties. Thus, one would predict that
only a limited subset of bacterial species would be able to become established on the host (habitat
filtering). However, the extent to which amphibian host factors dictate the selection, diversity, and
stability of the skin microbiota remains poorly understood. Moreover, we still lack knowledge about
how much variation in microbial community structure can be supported by host amphibian
genotypes. In other animals, it is clear that many host-specific factors can regulate the assembly of
their microbial communities. For example, previous studies in humans and laboratory mice have
shown that different genotypes support different microbiota (reviewed in Spor et al. 2011). Likewise,
in Nasonia wasps, ants and freshwater Hydra, species-specific microbiota emerge in
"phylosymbiotic" patterns that parallel speciation and ancestry (Brucker & Bordenstein, 2012; 2013;
Franzenburg et al., 2013; Sanders et al., 2014). In other animal systems genetic variation of immune

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Using multi-omics for probiotic selection

Associated genes have been associated with differences in symbiotic microbiota. For example, the gut microbial community structure of sticklebacks (Gasterosteus aculeatus) is correlated with the diversity of the individual’s Major Histocompatibility Complex (MHC) Class Ib genes (Bolnick et al., 2014). The MHC is a major set of adaptive immune genes that code for molecules that regulate recognition of foreign antigens and pathogens; however, the role of the MHC in regulating microbial communities is poorly understood. A proposed mechanism for MHC control of microbiota is that some MHC molecules may vary in their capacity to recognize the microbial motifs needed to mount an immune response against particular bacterial taxa (Bolnick et al., 2014). Microbes or their microbial antigens are taken up by professional antigen presenting cells such as dendritic cells and macrophages. The processed antigens are then presented as small peptides complexed with MHC to T lymphocytes. The T lymphocytes release cytokines that recruit other effector cells and they assist the development of antibodies.

In amphibians, several studies have demonstrated that the host microbiota in amphibians can vary by population (McKenzie et al. 2012; Kueneman et al. 2014; Walke et al., 2014), and it is possible that these differences correlate with population-level differences in immunogenetic diversity. Because the mucus of amphibians contains several classes of antibodies (Ramsey et al., 2010), it is likely that the antibodies expressed in the mucus would play a role in controlling which microbial species are allowed to colonize (Colombo et al., 2015).

In addition to the genetic diversity of genes involved in the adaptive immune system, amphibians produce a diverse array of innate immune defenses including antimicrobial peptides (AMPs), lysozymes and alkaloids (Macfoy et al., 2005; Conlon, 2011). A diverse array of AMPs are produced in amphibian’s granular glands such as brevins, ranatuerins, and magainins that are encoded by polymorphic genes that generate variation in peptide profiles among individuals (Tennessee & Blouin, 2007; Tennessen et al., 2009; Conlon, 2011; Daum et al., 2012). In comparison with the genetic diversity of MHC molecules, AMP genes and expressed peptides are much less diverse (Tennessee & Blouin, 2007). However, apparent gene duplications allow for gradual genetic changes that appear to be positively selected in response to pathogens (Tennessee & Blouin, 2007). Defensive AMPs appear to be released constitutively into the mucus at low concentrations, but they can be increased significantly when the amphibian hosts are alarmed or injured (Pask et al., 2012) and can be affected by environmental stressors (Katzenback et al. 2014). However, some amphibians appear to lack the capacity to produce conventional cationic AMPs (Conlon, 2011). Species that lack AMPs may be more dependent on other chemical factors present in the mucus (bacterial antifungal metabolites, lysozymes, and antibodies) that might affect assembly of the host-associated bacterial community, including inhibiting colonization and growth by skin pathogens.

In summary, all of the host mucosal chemical defenses (AMPs, lysozyme, alkaloids, and antibodies) have the potential to affect survival of some members of the community of skin bacteria. The interplay between chemical defenses in the mucus and microbial communities is not well understood. Future research is needed to understand to what extent microbes shape the immune compartment and how the immune compartment shapes the microbiome.

B. Biotic factors

Hosts are in constant contact with environmental microbial communities that serve as reservoirs. In the case of amphibian skin microbiota, environmental reservoirs may provide an important source of bacterial colonizers, which are needed since amphibian cutaneous microbial communities are frequently disturbed by skin shedding (Meyer et al., 2012). In humans, bacteria are found in deep
Using multi-omics for probiotic selection

epidermal layers, not only on the skin external layer (Nakatsuji et al., 2013), thus providing a
reservoir for re-inoculating the skin after disturbance. This has not yet been demonstrated for
amphibians: however, the salamander gut has been shown to be a reservoir for the anti-fungal
cutaneous bacterium *Janthinobacterium lividum* (Wiggins et al., 2011) and bacteria residing in gland
openings may also serve as a reservoir (Lauer et al., 2007).

Nonetheless, environmental reservoirs appear to be necessary to maintain the diversity of skin
symbiotic bacteria (Loudon et al., 2014a). For example, salamanders (*Plathodon cinereus*) without an
environmental bacterial reservoir showed a 75% decrease in bacterial richness, and their bacterial
communities became uneven with some OTUs becoming dominant in the majority of the individuals.
In contrast, salamanders that were housed with a soil reservoir maintained a greater bacterial
diversity that was more similar to naturally-associated communities (Loudon et al., 2014a). In the
case of red-eyed tree frogs (*Agalychnis callidryas*), individuals housed with plants had a greater
richness and abundance of skin bacteria than those housed without plants (Michaels et al., 2014).
These studies demonstrate that environmental reservoirs are necessary to maintain the diversity of
naturally-associated bacteria. In terms of probiotics, Muletz et al. (2012) demonstrated that *P.
cinereus* can acquire the beneficial bacterium *J. lividum* from soil, and salamanders that were able to
acquire *J. lividum* from the environment were less likely to be infected with *Bd* (Muletz et al., 2012).

The skin microbiota also interacts with invading skin pathogens such as *Bd*. In amphibians the skin
mucus contains a suite of microorganisms that may play a beneficial symbiotic role for the host
(Harris et al., 2006). Anti-*Bd* secretions from skin bacteria have been found on free-living hosts in
concentrations that inhibit *Bd in vitro* (Brucker et al., 2008; Becker et al., 2009). Furthermore, some
bacterially-produced metabolites interact synergistically and additively to inhibit *Bd* (Myers et al.
2012; Loudon et al., 2014a). Recent work has demonstrated that the composition and structure of
amphibian skin bacterial communities can change in response to *Bd* infection (Jani & Briggs, 2014).
However, it is still not well understood if changes in the diversity of the microbiota are accompanied
by changes in function (i.e., increases in the number of beneficial anti-*Bd* symbionts and therefore an
increased protective role of the skin microbiota).

A number of different *Bd* lineages have been identified and isolated from amphibian skin (Farrer et
al., 2011; Schloegel et al., 2012; Bataille et al., 2013), including the globally distributed and
hypervirulent global panzootic lineage (*BdGPL*) that has been associated with mass mortalities and
rapid population declines of amphibians (Farrer et al., 2011; 2013). Within the *BdGPL* lineage there
is considerable genetic variation, as well as significant differences in virulence between isolates
(Farrer et al., 2011; 2013). Many bacterial strains isolated from amphibian skin have the ability to
inhibit the growth of *Bd in vitro* (Harris et al., 2006; Woodham et al., 2014; Becker et al., 2015b,
Holden et al., 2015). However, it was recently demonstrated that bacteria differ in their capacity to
inhibit different *BdGPL* isolates, and only a small proportion of bacteria show broad scale inhibition
across the genetic variation exhibited by *BdGPL* (Antwis et al., 2015). This, coupled with the
variation in host response to different isolates of *BdGPL* (Farrer et al., 2011), means that potential
probiotics will need to account for differing virulence of *Bd* or that probiotics that show broad scale
inhibition must be identified and used.

In addition to the influence of environmental microbes and pathogens, the ecological interactions
among skin microbes would be expected to play a relevant role in structuring the skin microbiota.
Bacteria engage in the full breadth of ecological interactions, from antagonistic to facilitative
(reviewed by Faust & Raes, 2012). Many of these bacterial interactions are chemically mediated by
secondary metabolites, which can contribute to mutualistic interactions, such as cross-feeding or

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Using multi-omics for probiotic selection

Syntrophy, in which two species benefit from each other's metabolic products (Woyke et al., 2006; Faust & Raes, 2012; Loudon et al., 2014b). Secondary metabolite production is also influenced by the composition of the bacterial community (Onaka et al., 2011), and therefore changes in the community composition of the host (for example through environmental variation or diet) may intrinsically lead to changes in the secondary metabolite profile of the total community. In addition to mutualistic interactions, competition is common among microbes, and can occur via antibiotic production (Kelsic et al., 2015). Other forms of competition can range from occupying space and therefore inhibiting attachment of colonizing species, to more efficient consumption of shared resources.

C. Abiotic factors

The skin microbiome in vertebrates is highly sensitive to changes in humidity and temperature (Grice & Segre, 2011; Kueneeman et al., 2014). Therefore, the skin microbial community structure might be modified by exposure of the skin to different microclimates. This is particularly relevant for ectotherms like amphibians, in which habitat-mediated thermoregulation can expose the host (and its microbial symbionts) to a wide variety of microclimatic conditions over very short time periods (Huey, 1991). Moreover, seasonal variation may influence host behavior by increasing host body temperature (Rowley & Alford, 2013) and this could in turn modify the skin microbial structure. Warmer temperatures can increase the skin sloughing frequency of anurans, thus reducing the abundance of bacteria on the skin through frequent disturbance (Meyer et al., 2012; Ohmer et al., 2014). In addition, thermal conditions influence the activity and production of antifungal metabolites by symbiotic microbes on amphibian skin (Woodhams et al., 2014). For example, high temperatures can limit the production of antimicrobial metabolites, such as violacein and prodigiosin produced by \textit{J. lividum} strains (Schloss et al., 2010; Woodhams et al., 2014). However, for other bacterial probiotics, cooler temperatures may limit the production of antimicrobial products (Daskin et al., 2014). The combined influences of environmental variation on microbiome stability are poorly understood, and they likely vary among species from different habitats and ecosystems.

Moreover, temperature might also impact the skin microbiome by altering the interaction between the amphibian immune system and invading pathogens. Immunity in ectotherms is strongly affected by temperature (Raffel et al., 2006; Rollins-Smith et al., 2011; Rollins-Smith & Woodhams, 2012). In general, low temperatures (4-10°C) are predicted to favor \textit{Bd} (Woodhams et al., 2008; Voyles et al., 2012), and under these conditions amphibian immune defenses are delayed or diminished (Rollins-Smith et al., 2011; Rollins-Smith & Woodhams, 2012). In contrast, higher temperatures (25-30°C), nearer to the maximum for \textit{Bd} survival (Piotrowski et al., 2004; Stevenson et al., 2013), are predicted to favor the amphibian host, enabling them to develop a more effective immune response (Rowley & Alford, 2013). Similarly, \textit{Bsal} infections can be cleared by host exposure to 25°C for 10 days (Blooi et al., 2015). Thus, thermal preference of the host is associated with lower probabilities for \textit{Bd} or \textit{Bsal} infection (Rowley & Alford, 2013). In this respect, the skin microbiome may also be affected by pathogen invasions based on the host's and the pathogen's thermal preferences.

Conflict of interest statement

All authors declare that there are no conflicts of interests.

Author contributions

RH and ER contributed the original idea and outline. ER, RA, LB, MHB, MCB, RB, XH, AL, DM, KM, LR, JW, DW and RH contributed the initial writing of specific sections. ER, LB, MHB, MH, JK, VM, LR, SW, DW and RH contributed additional relevant ideas and sections as well as
Using multi-omics for probiotic selection

structuring the manuscript. ER integrated all sections and produced all drafts of the manuscript, and all authors edited several versions of the manuscript.

Funding

This project was funded by the NSF Dimensions in Biodiversity Program: DEB-1136602 to Reid N. Harris, DEB-1136640 to Lisa K. Belden and DEB-1136662 to Kevin PC. Minbiole. Bletz is supported by the German Academic Exchange Service (DAAD) and The German Research Foundation (DFG). Louise Rollins-Smith is supported by NSF grant IOS-1121758.

Figure legends

Figure 1. Main factors that influence the diversity and function of the amphibian skin microbiota, including host-associated factors, biotic factors, and abiotic factors (Box 1). Arrows in both directions indicate bidirectional interactions that might occur between the skin microbiota and a particular factor. AMPs stand for antimicrobial peptides. The size of each section is not proportional to the contribution of each of the factors.

Figure 2. Data showing proof of concept of a network analysis to identify correlations among bacterial and fungal OTUs on amphibian hosts. Network analyses depicting significantly correlated bacterial and fungal OTUs (SparCC r > 0.35). All square nodes represent OTUs (either bacteria or fungi). Red lines indicate negative correlations between two OTUs. Turquoise lines indicate positive correlations between two OTUs. A) Assessing directionality of interactions found between all bacteria and fungal taxa. Yellow = Betaproteobacteria, purple = Actinobacteria, blue = Fungi, white = other bacterial OTUs. B) Assessing directionality of interactions found between all bacterial interactions and Pathogen Bd. Yellow = bacteria that inhibit Bd in co-culture, blue = Unknown interaction with Bd in co-culture. Center of network = fungal pathogen Bd.

Figure 3. Flow diagram indicating the steps of the probiotic filtering protocol proposed by Bletz et al. (2013) that can be improved by omics data and integrative multi-omics analyses at different stages: (A) Section 5.1: Integrated multi-omics methods can inform the isolation probiotic candidates, (B) Section 5.2: probiotic candidates can be tested individually or in combination based on omics results and (C) Section 5.3: omics approaches can track the effect of probiotic bacteria on the host and on the environment. (D) Section 5.4: omics can provide probiotic candidates that can be tested in mucosose assays.

REFERENCES


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doi:10.1371/journal.pcbi.1002606.


This is a provisional file, not the final typeset article
Using multi-omics for probiotic selection


Using multi-omics for probiotic selection

Batrachochytrium dendrobatidis is inhibited by the cutaneous bacteria of amphibian species.

(2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME
J. 3, 818–824. doi:10.1038/ismej.2009.27.


(2015). Skin bacteria provide early protection for newly metamorphosed southern leopard frogs
(Rana sphenocephala) against the frog-killing fungus, Batrachochytrium dendrobatidis. Biol.

Bacteria isolated from bats inhibit the growth of Pseudogymnoascus destructans, the causative agent


doi:10.1016/j.ymeth.2015.03.017.

Jani, A. J., and Briggs, C. J. (2014). The pathogen Batrachochytrium dendrobatidis disturbs the frog
skin microbiome during a natural epidemic and experimental infection. Proc. Natl. Acad. Sci. 111,


(2014). Regulation of the Rana syrtica brevinin-1SY antimicrobial peptide during development and
in dorsal and ventral skin in response to freezing, anoxia and dehydration. J. Exp. Biol. 217, 1392–


http://dx.doi.org/10.1038/nature14485.


This is a provisional file, not the final typeset article
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Using multi-omics for probiotic selection


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Using multi-omics for probiotic selection


Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian


eukaryotes inhabiting our body as commensals or opportunistic pathogens. *Fungal Genom. Biol.* 4,

version*, 1(1).

Amphibian immune defenses against chytridiomycosis: impacts of changing environments. *Integr.

environment. In: Eco-Immunology. Demas, G.E. and Nelson, R.J. eds. Oxford University Press,
Oxford, United Kingdom. pp. 92-143.

doi:10.1371/journal.pone.0008694.

1238 Rowley, J. J. L., and Alford, R. A. (2013). Hot bodies protect amphibians against chytrid infection in

similarity analysis reveals unique associations among marine bacterioplankton species and
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1279 Statnikov, A., Henaff, M., Narendra, V., Konganti, K., Li, Z., Yang, L., et al. (2013). A
1280 comprehensive evaluation of multicategory classification methods for microbiomic data. Microbiome

1283 bacterial, archaean and protistan association networks reveal ecological linkages. ISME J. 5, 1414–


1288 transcriptomics reveals universal patterns of protein sequence conservation in natural microbial

1289 Stice, M. J., and Briggs, C. J. (2010). Immunization is ineffective at preventing infection and
1290 mortality due to the amphibian chytrid fungus Batrachochytrium dendrobatidis. J. Wildl. Dis. 46, 70–


1295 leads to gene duplication and low allelic variation. J. Mol. Evol. 65, 605–615. doi:10.1007/s00239-
1296 007-9045-5.

1298 (2009). Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (Rana
1299 pipiens) populations suggest intraspecies differences in resistance to pathogens. Dev. Comp.

1302 al. (2014). Nonlethal amphibian skin swabbing of cutaneous natural products for HPLC

1304 Underhill, D. M., and Iliev, I. D. (2014). The mycobionta: interactions between commensal fungi and


1311 Temperature alters reproductive life history patterns in Batrachochytrium dendrobatidis, a lethal
Using multi-omics for probiotic selection


doi:10.1073/pnas.0801921105.


doi:10.1038/ismej.2014.77.


doi:10.1016/j.brc.2007.05.004.


This is a provisional file, not the final typeset article
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