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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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47 **Abstract**
48 Emerging infectious diseases in wildlife are responsible for massive population declines. In
49 amphibians, chytridiomycosis caused by *Batrachochytrium dendrobatidis*, *Bd*, has severely affected
50 many amphibian populations and species around the world. One promising management strategy is
51 probiotic bioaugmentation of antifungal bacteria on amphibian skin. *In vivo* experimental trials using
52 bioaugmentation strategies have had mixed results, and therefore a more informed strategy is needed
53 to select successful probiotic candidates. Metagenomic, transcriptomic, and metabolomic methods,
54 colloquially called "omics", are approaches that can better inform probiotic selection and optimize
55 selection protocols. The integration of multiple omic data using bioinformatic and statistical tools and
56 *in silico* models that link bacterial community structure with bacterial defensive function can allow
57 the identification of species involved in pathogen inhibition. We recommend using 16S rRNA gene
58 amplicon sequencing and methods such as indicator species analysis, the K-S Measure, and co-
59 occurrence networks to identify bacteria that are associated with pathogen resistance in field surveys
60 and experimental trials. In addition to 16S amplicon sequencing, we recommend approaches that give
61 insight into symbiont function such as shotgun metagenomics, metatranscriptomics or metabolomics
62 to maximize the probability of finding effective probiotic candidates, which can then be isolated in
63 culture and tested in persistence and clinical trials. An effective mitigation strategy to ameliorate
64 chytridiomycosis and other emerging infectious diseases is necessary; the advancement of omic
65 methods and the integration of multiple omic data provide a promising avenue toward conservation
66 of imperiled species.
67
68 **1. Introduction**
69
70 Emerging infectious diseases (EIDs) in wildlife pose a grave threat to biodiversity (Wake &
71 Vredenburg, 2008; Blehert et al., 2009; Fisher et al., 2009; 2012; Price et al., 2014; Schrope, 2014).
72 Examples of EIDs caused by fungal pathogens include white-nose syndrome in bats (Blehert et al.,
73 2009) and chytridiomycosis in amphibians (Berger et al., 1998). The latter, caused by
74 *Batrachochytrium dendrobatidis* (*Bd*), is considered the greatest disease threat to biodiversity at the
75 current time (Wake & Vredenburg, 2008). Recently, a newly described chytrid fungal species,
76 *Batrachochytrium salamandrivorans* (*Bsal*) has been identified as the causal agent of
77 chytridiomycosis in salamanders and is causing many salamander populations declines in Europe
78 (Martel et al., 2013; 2014; Yap et al., 2015). Several strategies have been proposed to contend against
79 EIDs in amphibians (Fisher et al., 2012; Woodhams et al., 2012; McMahon et al., 2014; Langwig et
80 al., 2015) including vaccination, selective breeding and the use of probiotic bioaugmentation (Harris
81 et al., 2006; Woodhams et al., 2007; Harris et al., 2009; Stice & Briggs, 2010; McMahon et al., 2014;
82 Hoyt et al., 2015). Successful implementation of these approaches for the conservation of wild
83 populations will benefit from further laboratory and field-testing, particularly when informed by
84 integrated multi-omics methods.
85
86 There is growing evidence that probiotic therapy in particular could be a promising approach to
87 mitigating disease in a variety of organisms, including human, plant crops -and wildlife systems
88 (Harris et al., 2009; Sánchez et al., 2013; Akhter et al., 2015; Forster & Lawley 2015; Hoyt et al.,
89 2015; Papadimitriou et al., 2015). A relevant case comes from studies on plant-microbial
90 interactions, which have identified several bacterial taxa involved in protection of plant crops against
91 pathogens and extreme environmental stressor as well as in nutrient availability (Berlec 2012). Some
92 of these microorganisms have been widely and successfully used as bio-fertilizers or biocontrols in
93 plant agriculture (Bhardwaj et al., 2014; Lakshmanan et al., 2014). In amphibians, the approach that
94 is currently being developed is probiotic bioaugmentation, which is the establishment and
95 augmentation of protective ~~bacteria~~ microbes that are already naturally occurring on at least some

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 & Siegre, 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; Buffie et al., 2015; Forster & Lawley et al., 2015), we focus on applying these principles to the amphibian system, emphasizing current~~ly 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

144 success of probiotic selection supported by multi-omics data. It is important to note that the omics
145 methods as well as the statistical, modeling and integrative methods mentioned in this review are
146 only a subset of the current methods available and should therefore not be considered the only
147 methods researchers can use to identify successful probiotic candidates.
148
149 **2. Ecology of the amphibian skin microbiome**
150
151 The amphibian skin microbiome is defined as the microbiota and its combined genetic material
152 present on the skin. Determining the main drivers of the assembly of the skin microbiome through
153 the use of omic methods and culture-dependent approaches may greatly enhance our ability to
154 develop successful probiotic treatments and prevent amphibian population declines caused by
155 chytridiomycosis.
156
157 The amphibian skin microbiome is determined by the microbiota's interactions with host-associated
158 factors and with abiotic and biotic factors (Figure 1, Box 1). Host-associated factors include host
159 genetic diversity and the adaptive and innate immune systems (Box 1A), in addition to host behavior,
160 ecology and development. Biotic factors include ecological interactions between skin symbiotic
161 microbes and the microbial composition of environmental reservoirs (Box 1B), and abiotic factors
162 include environmental conditions such as temperature and humidity (Box 1C). Altogether, the factors
163 that influence the skin microbiome of amphibians determine the chemical composition of the skin
164 mucus and in turn help determine the degree of host susceptibility against pathogens (Searle et al.,
165 2011; Woodhams et al., 2014). Box 1 summarizes the current state of knowledge on the drivers of the
166 amphibian skin microbiome. We now focus on omics and integrative multi-omics methods and how
167 they can be used to address knowledge gaps that are key to developing effective probiotic strategies.
168
169 **3. Omics methods to identify probiotic candidates**

170 An important first step toward the identification of potential probiotics in amphibians is to determine
171 differences in the structure and function of skin microbial communities in the presence or absence of
172 *Bd*. This approach includes comparing diseased and not diseased individuals after exposure to *Bd* in
173 laboratory trials, as well as examining *Bd*-tolerant or resistant species from localities that have
174 experienced population declines. The assumption is that individuals or species that persist in the
175 presence of *Bd* might harbor protective bacteria that allowed them to survive. Such studies can be
176 done through 16S amplicon sequencing of the whole microbial community (Caporaso et al., 2012).
177 Furthermore, in order to identify key bacterial species responsible for pathogen protection it will be
178 necessary to go beyond taxonomic descriptions and determine the functional capacities of the
179 community through the use of additional techniques such as shotgun metagenomics,
180 metatranscriptomics and metabolomics. These approaches can be used to identify bacterial strains
181 that contain genes whose function could make them an effective probiotic. For example, based on
182 previous knowledge about bacterial interactions, searching for genes associated with the production
183 of antibiotics (including anti-fungal metabolites) and beneficial host-microbe interactions could
184 increase the chances of selecting good probiotic candidates.
185 It is important to emphasize that the use of omic approaches to identify probiotics will only be
186 relevant if these are linked with biological assays of culturable bacteria (*sensu* Becker et al., 2015b).
187 Linking culture-independent with culture-dependent data is a fundamental step towards the
188 identification of successful probiotics (Walke et al., 2015). Importantly, if bacterial cultures with
189 inhibitory activities are available, then physiological, metabolic and genomic analyses of these strains
190 can greatly inform omics predictions. Below, we describe currently available omic approaches and
191 their applications for probiotic selection in amphibians.

192 **3.1. 16S amplicon sequencing**

193 Amplicon sequencing is the sequencing of a particular gene or gene fragment of an entire microbial
194 community through the use of high-throughput sequencing methods (Metzker 2010). In particular,
195 16S amplicon sequencing of skin bacterial communities has allowed us to determine the most
196 prevalent and relatively abundant bacterial OTUs (operational taxonomic units) on different
197 amphibian species and populations, and across life-history stages (McKenzie et al., 2012, Kueneman
198 et al., 2014, Loudon et al., 2014a, Walke et al., 2014). By providing information about which
199 bacterial taxa appear to be involved in pathogen protection, 16S amplicon sequencing can help target
200 the isolation of potential probiotic bacteria in pure culture. This can be accomplished using data from
201 both field surveys and laboratory experiments.

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

209 Field surveys, however, are inadequate to determine causal relationships between OTU presence and
210 pathogen presence. Experimental laboratory *Bd* exposures are essential to determine changes in the
211 microbial structure in response to *Bd* infection so they can provide information about which bacterial
212 taxa could be involved in host defense against pathogens. Variation in susceptibility to *Bd* has been
213 linked to changes in cutaneous bacterial community structure (Becker et al. 2015a, Holden et al.,
214 2015), presence of skin antifungal metabolites (Brucker et al., 2008; Becker et al. 2009; Becker et al.
215 2015b), function of the mucus components (Woodhams et al., 2014), and MHC genotype (Savage &
216 Zamudio, 2011, Bataille et al., 2015). For example, an experiment investigating the use of probiotics
217 to prevent chytridiomycosis in the highly susceptible Panamanian golden frog (*Atelopus zeteki*)
218 demonstrated that individuals that were able to clear *Bd* infection harbored a unique community of
219 bacteria on their skin prior to probiotic treatment (Becker et al., 2015a). Furthermore, the authors
220 identified several bacterial families on surviving frogs that were correlated with clearance of *Bd*
221 (Flavobacteriaceae, Sphingobacteriaceae, Comamonadaceae and Rhodocyclaceae). In contrast OTUs
222 on individuals that died belonged to the families Micrococcineae, Rhizobiaceae, Rhodobacteraceae,
223 Sphingomonadaceae and Moraxellaceae (Becker et al., 2015a). To determine if the OTUs associated
224 with survival could prevent chytridiomycosis in *A. zeteki*, the next steps would be to isolate the
225 potentially beneficial OTUs from surviving golden frogs, test the isolates for *Bd* inhibition *in vitro*
226 (Bell et al., 2013) and/or using mucosome assays (Woodhams et al., 2014), and finally test the
227 resistance of inoculated individuals to *Bd* infection (Bletz et al., 2013). In addition, the ability to
228 predict host susceptibility via 16S amplicon sequencing may provide a useful tool for captive
229 population managers to identify individuals that could be used for reintroduction trials.
230

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

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 can~~ould~~ also unravel functional responses involved in bacterial-host interactions such as the expression of adhesin 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 amphibians 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

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 transc~~i~~riptomic studies on amphibian hosts (Ellison et al., 2014, Savage et al., 2014; Price et al., 2015) and in parallel measure changes in the skin microbial structure~~ome~~ (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 microbially-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).

332
333 **4. Statistical tools to identify probiotic candidates and integrate multi-omics data.**

334 **4.1. Identifying key bacterial species associated with amphibian survival against *Bd***

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

345 An additional statistical technique is the K-S Measure (Loftus et al., 2015), which is an extension of
346 the Kolmogorov-Smirnov (K-S) test statistic (Kolmogorov 1933, Smirnov 1936). While the K-S test
347 statistic has long been used to assess differences in empirical distribution functions between two
348 groups, the K-S Measure was designed to assess differences in the distributions of the relative
349 abundances of individual OTUs among K > 2 groups. For a given OTU, empirical relative abundance
350 distribution functions are assembled for each group using the data for all individuals assigned to that
351 group. The K-S Measure simultaneously assesses the magnitude of the differences between the
352 distributions, using the weighted sum of the K-S statistics for all pairwise comparisons of
353 distributions defined by K groups. The K-S Measure ranges from zero to one, where values closer to
354 one imply greater differences between the K distributions than values closer to zero (Loftus et al.,
355 2015).
356
357 The linear discriminant analysis (LDA) effect size method, LEfSe, can also be an informative method
358 (Segata et al., 2011). LEfSe can be used to compare among groups that are biologically relevant and
359 determine which features (organisms, clades, OTUs, genes, or functions) are significantly different
360 (Albanese et al., 2015, Clemente et al., 2015, Zeng et al., 2015). LEfSe determines the factors that
361 most likely explain differences between classes by coupling standard tests for statistical significance
362 (Kruskal Wallis and Wilcoxon non parametric tests) with additional discriminant tests that estimate
363 the magnitude of the effect (LDA score).
364
365 Another promising analysis technique is DESeq2, which offers higher power detection for smaller
366 sample sizes (less than 20 samples per group) compared to traditional non-parametric tests based on
367 Kruskal-Wallis and Wilcoxon rank-sum approaches (McMurdie & Holmes, 2014; Weiss et al.,
368 2015). While the non-parametric tests do not assume a distribution, DESeq2 assumes a negative
369 binomial distribution to obtain maximum likelihood estimates for a feature's (gene, OTU, etc.) log-
370 fold change between two groups (Anders & Huber, 2010; Love et al. 2013). Bayesian shrinkage is
371 then used to reduce the log-fold change toward zero for those OTUs of lower mean count and/or with
372 higher dispersion in their count distribution. These shrunken log-fold changes are tested for
373 significance with a Wald test. If the average number of sequences per sample between the two
374 sample groups differs greatly (>3x), it is better to use a Kruskal-Wallis type approach such as LEfSe
375 for lower type 1 error. All methods, indicator species, K-S Measure, LEfSe and DESeq2, take into
376 account the relative abundance and prevalence of each OTU with the latter two methods allowing for
377 a stratified statistical design with biologically relevant classes and subclasses.

378
379 We suggest that all or some of these statistical methods can be used in parallel to identify taxa
380 involved in protection against pathogens. Importantly, some of these statistical tools can be used to
381 identify OTUs based on 16S amplicon sequencing but they can also be used to identify genes and
382 metabolites associated with pathogen protection based on metagenomics, metatranscriptomics and
383 metabolomics data (Segata et al., 2011, Love et al., 2013; Loftus et al., 2015; Weiss et al., 2015).
384
385 **4.2. Defining interactions and networks involved in protection against pathogens**
386
387 One inherent challenge of omic data is interpreting the complex interactions present within the data
388 collected. Many microbial datasets can have more than 5,000 features (e.g., OTUs in the case of 16S
389 amplicon sequencing), so this implies almost 12.5 million possible two-feature correlations. Also, it
390 is expected that within these complex microbial communities three or more feature interactions will
391 occur. Furthermore, omic datasets exhibit diverse challenges, including only providing relative
392 abundances based on a fixed total number of sequences rather than absolute abundances, or the
393 abundance and spacial distribution of zeroes in a data matrix (compositionality) (Aitchison, 1986;
394 Lovell, 2010; Friedman & Alm, 2012). Data sets with many zeroes, missing data due to incomplete
395 sampling, and ecological relationship diversity (e.g. parasitism, commensalism, etc.) further
396 complicates statistical analysis (Reshef et al 2011; Friedman & Alm, 2012). However, despite the
397 challenges, computation is possible in terms of time and expense as compared with evaluating more
398 than 12.5 million microbial interactions in the laboratory. Also, the mathematical and statistical
399 approaches for analyzing community data are improving. One technique for inferring microbial
400 interactions from sequencing data is correlation network analysis. Networks consist of “nodes”
401 (OTUs, genes, metabolites, integrated omics) and “edges”, based on the strength of the interaction
402 between nodes, and which imply a biologically or biochemically meaningful relationship between
403 features (Imangaliyev et al., 2015). Interaction values between nodes are commonly referred to as co-
404 occurrence patterns (Faust & Raes, 2012).
405
406 Many different techniques have been developed for assessing correlations and constructing
407 interaction networks. Some classic correlation techniques are the Pearson correlation coefficient
408 (Pearson 1909), which assess linear relationships, or the Spearman correlation coefficient (Spearman
409 1904), which measures ranked relationships. Both Pearson and Spearman correlation are very useful
410 (e.g. Arumugam et al., 2011; Barberan et al., 2012a; Buffie et al., 2015), however neither was
411 developed specifically for the challenges of sequencing data, e.g. compositionality. Of the two,
412 Spearman is less adversely affected by the former challenges. Other correlation methods that have
413 been developed include CoNet (Faust et al., 2012), MENA, or Molecular Ecological Network
414 Analysis (Zhou et al., 2011; Deng et al., 2012), Maximal Information Coefficient (MIC) (Reshef et
415 al., 2011), Local Similarity Analysis (LSA) (Beman et al., 2011, Ruan et al., 2006, Steele et al., 2011,
416 Xia et al., 2013), and Sparse Correlations for Compositional Data (SparCC) (Friedman & Alm,
417 2012). Network visualizations are often performed in the igraph package in R (R Core Team, 2014)
418 or in Cytoscape (Shannon, et al., 2003).
419
420 For probiotic selection, the construction and analysis of networks can infer which taxa occur together
421 in natural communities, and can attempt to identify the direction of interactions between taxa or
422 groups of highly connected taxa (Barberán et al. 2012a). For example, correlation networks in
423 human and mouse models helped identify *Clostridium scindens* as exhibiting a negative correlation
424 pattern with the pathogen *Clostridium difficile*. Transfer of *C. scindens,* either alone or with other
425 bacteria identified by the correlation networks, was then experimentally shown to increase resistance
426 to *C. difficile* infection in mouse models (Buffie et al., 2015). In the case of amphibians, networks

427 that integrate bacterial and fungal omics data taken from hosts, can inform our understanding of
428 interactions occurring between diverse bacterial and fungal taxa (Figure 2A). Determining the
429 negative or positive correlations that shift in the presence of a pathogen like *Bd* in experimental trials
430 could help distinguish groups of microbes (mainly bacteria and fungi) involved in resistance against
431 pathogens (Figure 2B).
432
433 In order to identify potential probiotics against *Bd* in amphibians, correlation networks can be used to
434 compare individual or group interactions in omic data between (1) *Bd*-positive and *Bd*-negative
435 populations in the field, (2) *Bd*-infected and uninfected hosts in experimental trials, (3) hosts with
436 differential *Bd* infection intensity in the field or in experiments and (4) hosts from different life
437 stages. However, caution is warranted when inferring a mechanism of interaction based solely on
438 patterns of correlation (Levy & Borenstein, 2013). Targeted culturing of taxa identified by networks
439 may additionally be used to inform probiotic selection and test their ability to inhibit *Bd* singly or
440 jointly (see Section 5.2), as there may be synergistic *Bd* inhibition (Loudon et al., 2014b). These taxa
441 can be the basis for forming specific hypotheses that can be explored in experimental studies such as
442 a probiotic treatment to determine if the addition of these species to amphibian skin can establish,
443 persist, and increase the anti-*Bd* function of the microbial community of susceptible species.
444
445 **4.3. Integrating multi-omics data to identify anti-fungal genetic or metabolic pathways**
446
447 Metagenomics, metatranscriptomics and metabolomics are important tools to determine molecular
448 pathways present in microbial ecosystems. One of the main goals is integrating these multiple
449 massive data sets to distinguish community patterns associated with a specific function such as host
450 disease resistance. Protection against *Bd* in amphibians is likely achieved by a combination of
451 functional pathways present in the skin microbiome in concert with the host's immune system.
452 Therefore, the integration of multiple high dimensional datasets using predictive computational
453 approaches such as bioinformatic predictive tools, multi-omic correlations and *in silico* models are
454 key to predict functional outcomes within the skin microbiome (Borenstein, 2012; Langille et al.,
455 2013; McHardy et al., 2013; Meng et al., 2014). One approach termed Reverse Ecology, offers a
456 promising way to use high-throughput genomic data to infer ecological interactions from complex
457 biological systems (Levy and Borenstein, 2012; 2014). It involves predicting the metabolic capacity
458 of a biological system (including symbiotic systems) based on metagenomic data through the use of
459 graph-theory based algorithms and genome-scale metabolic networks (Borenstein et al., 2008;
460 Borenstein & Feldman, 2009; Freilich et al., 2009; Levy & Borenstein, 2012, Manor et al., 2014). To
461 date, the amphibian skin microbiome has mainly been described using culture-dependent techniques
462 and 16S amplicon sequencing. The use of these additional techniques may greatly improve our
463 understanding of this microbial system and could allow us to identify fundamental metabolic
464 pathways and ecological networks associated with defense against pathogens like *Bd*. For example,
465 multi-omic correlations of 16S amplicon sequencing and metabolomics (McHardy et al., 2013) may
466 allow us to determine bacterial taxa and metabolites associated with *Bd* inhibition in *Bd*-tolerant
467 species and in individuals exposed to *Bd* in experimental trials.

468 Moreover, the bacterial taxa that produce the metabolites could be determined by statistical methods
469 that associate metabolite presence with bacterial species' presence. For example, using random forest
470 with machine learning one can rank microbes by relative contribution (importance) (Knights et al.,
471 2011a; 2011b; 2011c; Ditzler et al. 2014). Random forest is an accurate machine-learning multi-
472 category classification algorithm for linking abundances of microbial taxa to physiological states
473 such as metabolite production or immune function (Statnikov et al. 2013). Bacterial species that

produce one or more anti-*Bd* metabolites that are associated with survival in a *Bd*-positive environment would be excellent probiotic candidates for bioaugmentation in at-risk populations.

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

Bletz et al. (2013) recently outlined sampling strategies and screening protocols for identifying ideal probiotics for amphibians (Figure 3). The framework involves (1) collecting and culturing skin microbes from selected host species; (2) isolating all morphologically-distinct colonies into pure culture; (3) testing each isolate for its ability to inhibit *Bd in vitro* (Bell et al., 2013); (4) testing highly inhibitory isolates for their ability to colonize and persist on amphibian skin; (5) and for those isolates that persist testing their ability to protect the host against *Bd* infection in clinical trials in the laboratory, followed by field trials (Bletz et al., 2013). In the case of the skin of some amphibian species, the dominant members of the microbiota are readily cultured (Walke et al. 2015), whereas some rare but prevalent members identified by 16S amplicon sequencing have been difficult to isolate in culture (Loudon et al. 2014a). Specialized media may be necessary to target microbes identified by omics approaches including not only bacteria but also fungi. Even though most of the probiotic search in amphibians has focused on bacterial candidates, the filtering protocol proposed by Bletz et al., (2013) could be also used to target potential fungal probiotics. Omic datasets and the integration of multi-omic analyses can facilitate the selection of the probiotic candidates that progress through this sampling and screening protocol. Below we describe the steps of the filtering protocol (Bletz et al., 2013) and the mucosome assay (Woodhams et al., 2014) that can be improved by omics and the integration of multi-omic approaches (Figure 3).

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

A probiotic approach typically requires culturing and isolation of microbial species in order to test their antifungal functions and use only those species with desired properties. Given the taxonomic diversity ~~of the bacterial component~~ of the amphibian skin microbiome (Kueneman et al., 2014; Loudon et al., 2014a, Walke et al., 2014; Becker et al., 2015a; Kueneman et al., 2015), it would be useful to reduce the number of ~~bacteria~~ microorganisms that one is trying to isolate and test for inhibition. Omics methods can streamline the ~~bacterial~~ isolation process by identifying promising probiotic taxa, which can then be isolated using media and culture conditions that favor or enrich for specific bacterial or fungal groups (Watve et al., 2000; Connon & Giovannoni, 2002; Rappé et al., 2002; Zengler et al., 2002, Vartoukian et al., 2010).

Through the integration of multi-omics data, ~~bacterial~~ microbial community members that are associated with surviving amphibian populations in the field and in laboratory experiments can be identified. We suggest using several methods such as indicator species analysis, the K-S Measure, LEfSe and co-occurrence networks in parallel to identify probiotic candidates. The main goal of using several methods is to obtain a list of OTUs that are congruent among methods. OTUs suggested as probiotic candidates by these culture-independent methods can be matched to bacterial isolates in pure culture identified with 16S rRNA Sanger sequencing (Woodhams et al., 2015) or to bacterial strains whose whole genome has been sequenced. These isolates would then proceed to testing for inhibition against *Bd* using *in vitro* challenge assays (Bell et al., 2013) or mucosome assays (Woodhams et al., 2014).

Commented [B1]: Insert refrences for this

521 **5.2. Using omics to predict if probiotic candidates should be tested individually or in**
522 **combination.**

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

527 Single isolate probiotics have been successful in some systems such as the probiotic bacterium *J.*
528 *lividum* in experimental trials with the host *R. muscosa* experimental trials (Harris et al., 2009).
529 However, research in several symbiotic systems has shown that bacterial mixtures are necessary to
530 exert a protective effect against pathogens and a restorative effect on hosts (Lawley et al., 2012;
531 Fraune et al., 2014). For example, in a mouse model of *C. difficile* infection, a six-species probiotic
532 mixture led to a community reset *and* recovery from *C. difficile* infection (Lawley et al., 2012). The
533 authors speculated that the six species in combination were successful due to their phylogenetic
534 distinctiveness, which allowed them to more effectively fill available niche space. Importantly each
535 species alone was not curative, but each species was necessary in the mixture for the treatment to be
536 effective (Lawley et al., 2012).
537

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

544
545 **5.3. Using omics data to track the effectiveness of probiotic bacteria in laboratory and field**
546 **trials.**
547

548 Omics can be used to determine if a probiotic was able to colonize, persist and/or trigger antifungal
549 pathways in the symbiotic community. This is key to its success as a probiotic (Bletz et al., 2013).
550 We hypothesize that this can be accomplished if the community reaches an alternative stable state
551 once the candidate taxon is applied and community structure begins to shift in response (Faust &
552 Raes, 2012; Fierer et al., 2012). The new stable state of the community must have antifungal
553 functions and sufficient competitive abilities against invading pathogens to protect the host. Co-
554 occurrence networks could be helpful to track whether bacterial interactions remain stable or shift
555 through time after probiotic application (Rosvall & Bergstrom, 2010). In addition, these approaches
556 could be useful for understanding whether probiotics applied during one life stage persist and remain
557 effective in subsequent life stages (e.g., through metamorphosis).
558

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

567 of both the host organism and microbial systems in the surrounding environment to probiotic
568 application.
569
570 **5.4. Using omics approaches to inform probiotic testing in mucosome assays**
571
572 The integrated defenses of the amphibian skin mucus, including antimicrobial peptides, microbiota,
573 mucosal antibodies, lysozymes and alkaloid secretions, are called the mucosome. A mucosome assay
574 developed by Woodhams et al. (2014) can be used to predict the infection prevalence of *Bd*-exposed
575 populations and the survival outcome upon exposure. Briefly, a mucosome assay consists of placing
576 individuals in a bath that collects their mucosal secretions. The secretions are used for *in vitro*
577 viability assays, in which they are tested for their ability to kill *Bd*. This assay accurately predicts
578 adult amphibian survival upon *Bd* exposure (Woodhams et al., 2014).
579
580 Importantly, the mucosome assay can be used to measure and predict the effectiveness of probiotic
581 treatments. Probiotic candidates identified by omics analyses need to be isolated through culturing
582 methods and then added to amphibian skins to evaluate their effectiveness using a mucosome assay.
583 This is accomplished by comparing the mucosome function before and after the addition of a
584 probiotic bacterium or a group of probiotic bacteria. Probiotic candidates that pass the preliminary
585 assay screen would then be ready for persistence and clinical trials (Figure 3D). The advantage of
586 using the mucosome assay is that it could minimize the need to expose amphibians to *Bd* in clinical
587 trials, which is particularly relevant in the case of endangered species or species that are naïve to the
588 disease.
589
590 **6. Important considerations and future directions**
591
592 To facilitate the identification of successful probiotic candidates, we recommend using an
593 interdisciplinary approach. The interaction and collaboration of scientists who have different
594 expertise as well as interaction with natural resource managers can greatly improve the outcomes of
595 probiotic research.
596
597 In addition to probiotic therapy for the reintroduction of species currently being held in captivity, one
598 important challenge is to identify probiotic candidates for species that are still naïve to pathogen
599 infections. Two relevant cases from highly diverse regions are frogs in regions of Madagascar that
600 have not been exposed to *Bd* (Bletz et al., 2015) and North American salamanders that are so far
601 naïve to *Bsal* (Martel et al., 2013; 2014; Yap et al., 2015).
602
603 Omic methods, along with mucosome assays and culture-dependent methods, may greatly improve
604 our knowledge on the capacity of ~~naïve individuals~~amphibians ~~-~~(and their microbiomes) to contend
605 against ~~novel~~ pathogenic infectionss~~s~~. In addition, other non-omic techniques such as real-time PCR
606 (qPCR), fluoresence *in situ* hybridization (FISH) and mass spectometry of culturable communities
607 may greatly inform probiotic discovery since they can increase our understanding of the amphibian
608 skin microbiome dynamics (Watrous et al., 2012; Barea et al., 2015). ~~This is a fundamental step for~~
609 ~~developing effective probiotics that could be applied to at-risk amphibian populations.~~
610
611 ~~In addition to~~Based on previous research on the amphibian system, this review has mainly focused on
612 bacterial probiotics. However~~,~~ future research may benefit by considering the micro-eukaryotic and
613 viral components of the skin community. Indeed, fungi are important components of mammalian and
614 amphibian skin (Underhill et al., 2014; Kueneman et al., 2015), and viruses have been linked to
615 dysbiosis in the oral cavity (Edlund et al., 2015). Several studies have examined the importance of

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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 Bsal*. 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 (Kesarcodi-Watson et al., 2008; ~~Ringø et al., 2010;~~ Bhardwaj et al., 2014; Lakshmanan et al., 2014Hoyt 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

665 associated genes has been associated with differences in symbiotic microbiota. For example, the gut
666 microbial community structure of sticklebacks (*Gasterosteus aculeatus*) is correlated with the
667 diversity of the individual's Major Histocompatibility Complex (MHC) Class IIb genes (Bolnick et
668 al., 2014). The MHC is a major set of adaptive immune genes that code for molecules that regulate
669 recognition of foreign antigens and pathogens; however, the role of the MHC in regulating microbial
670 communities is poorly understood. A proposed mechanism for MHC control of microbiota is that
671 some MHC molecules may vary in their capacity to recognize the microbial motifs needed to mount
672 an immune response against particular bacterial taxa (Bolnick et al., 2014). Microbes or their
673 microbial antigens are taken up by professional antigen presenting cells such as dendritic cells and
674 macrophages. The processed antigens are then presented as small peptides complexed with MHC to
675 T lymphocytes. The T lymphocytes release cytokines that recruit other effector cells and they assist
676 the development of antibodies.
677
678 In amphibians, several studies have demonstrated that the host microbiota in amphibians can vary by
679 population (McKenzie et al. 2012; Kueneman et al. 2014; Walke et al., 2014), and it is possible that
680 these differences correlate with population-level differences in immunogenetic diversity. Because the
681 mucus of amphibians contains several classes of antibodies (Ramsey et al 2010), it is likely that the
682 antibodies expressed in the mucus would play a role in controlling which microbial species are
683 allowed to colonize (Colombo et al., 2015).
684
685 In addition to the genetic diversity of genes involved in the adaptive immune system, amphibians
686 produce a diverse array of innate immune defenses including antimicrobial peptides (AMPs),
687 lysozymes and alkaloids (Macfoy et al., 2005; Conlon, 2011). A diverse array of AMPs are produced
688 in amphibian's granular glands such as brevinins, ranatuerins, and magainins that are encoded by
689 polymorphic genes that generate variation in peptide profiles among individuals (Tennessen &
690 Blouin, 2007; Tennessen et al., 2009; Conlon, 2011; Daum et al., 2012). In comparison with the
691 genetic diversity of MHC molecules, AMP genes and expressed peptides are much less diverse
692 (Tennessen & Blouin, 2007). However, apparent gene duplications allow for gradual genetic changes
693 that appear to be positively selected in response to pathogens (Tennessen & Blouin, 2007). Defensive
694 AMPs appear to be released constitutively into the mucus at low concentrations, but they can be
695 increased significantly when the amphibian hosts are alarmed or injured (Pask et al., 2012) and can
696 be affected by environmental stressors (Katzenback et al. 2014). However, some amphibians appear
697 to lack the capacity to produce conventional cationic AMPs (Conlon, 2011). Species that lack AMPs
698 may be more dependent on other chemical factors present in the mucus (bacterial antifungal
699 metabolites, lysozymes, and antibodies) that might affect assembly of the host-associated bacterial
700 community, including inhibiting colonization and growth by skin pathogens.
701
702 In summary, all of the host mucosal chemical defenses (AMPs, lysozyme, alkaloids, and antibodies)
703 have the potential to affect survival of some members of the community of skin bacteria. The
704 interplay between chemical defenses in the mucus and microbial communities is not well understood.
705 Future research is needed to understand to what extent microbes shape the immune compartment and
706 how the immune compartment shapes the microbiome.
707
708 **B. Biotic factors**
709
710 Hosts are in constant contact with environmental microbial communities that serve as reservoirs. In
711 the case of amphibian skin microbiota, environmental reservoirs may provide an important source of
712 bacterial colonizers, which are needed since amphibian cutaneous microbial communities are
713 frequently disturbed by skin shedding (Meyer et al., 2012). In humans, bacteria are found in deep

714 epidermal layers, not only on the skin external layer (Nakatsuji et al., 2013), thus providing a
715 reservoir for re-inoculating the skin after disturbance. This has not yet been demonstrated for
716 amphibians; however, the salamander gut has been shown to be a reservoir for the anti-fungal
717 cutaneous bacterium *Janthinobacterium lividum* (Wiggins et al., 2011) and bacteria residing in gland
718 openings may also serve as a reservoir (Lauer et al., 2007).
719
720 Nonetheless, environmental reservoirs appear to be necessary to maintain the diversity of skin
721 symbiotic bacteria (Loudon et al., 2014a). For example, salamanders (*Plethodon cinereus*) without an
722 environmental bacterial reservoir showed a 75% decrease in bacterial richness, and their bacterial
723 communities became uneven with some OTUs becoming dominant in the majority of the individuals.
724 In contrast, salamanders that were housed with a soil reservoir maintained a greater bacterial
725 diversity that was more similar to naturally-associated communities (Loudon et al., 2014a). In the
726 case of red-eyed tree frogs (*Agalychnis callidryas)*, individuals housed with plants had a greater
727 richness and abundance of skin bacteria than those housed without plants (Michaels et al., 2014).
728 These studies demonstrate that environmental reservoirs are necessary to maintain the diversity of
729 naturally-associated bacteria. In terms of probiotics, Muletz et al. (2012) demonstrated that *P.*
730 *cinereus* can acquire the beneficial bacterium *J. lividum* from soil, and salamanders that were able to
731 acquire *J. lividum* from the environment were less likely to be infected with *Bd (*Muletz et al., 2012).
732
733 The skin microbiota also interacts with invading skin pathogens such as *Bd*. In amphibians the skin
734 mucus contains a suite of microorganisms that may play a beneficial symbiotic role for the host
735 (Harris et al., 2006). Anti-*Bd* secretions from skin bacteria have been found on free-living hosts in
736 concentrations that inhibit *Bd in vitro* (Brucker et al., 2008; Becker et al., 2009). Furthermore, some
737 bacterially-produced metabolites interact synergistically and additively to inhibit *Bd* (Myers et al.
738 2012; Loudon et al., 2014a). Recent work has demonstrated that the composition and structure of
739 amphibian skin bacterial communities can change in response to *Bd* infection (Jani & Briggs, 2014).
740 However, it is still not well understood if changes in the diversity of the microbiota are accompanied
741 by changes in function (i.e., increases in the number of beneficial anti-*Bd* symbionts and therefore an
742 increased protective role of the skin microbiota).
743
744 A number of different *Bd* lineages have been identified and isolated from amphibian skin (Farrer et
745 al., 2011; Schloegel et al., 2012; Bataille et al., 2013), including the globally distributed and
746 hypervirulent global panzootic lineage (*Bd*GPL) that has been associated with mass mortalities and
747 rapid population declines of amphibians (Farrer et al., 2011; 2013). Within the *Bd*GPL lineage there
748 is considerable genetic variation, as well as significant differences in virulence between isolates
749 (Farrer et al., 2011; 2013). Many bacterial strains isolated from amphibian skin have the ability to
750 inhibit the growth of *Bd in vitro* (Harris et al., 2006; Woodhams et al., 2014; Becker et al., 2015b,
751 Holden et al., 2015). However, it was recently demonstrated that bacteria differ in their capacity to
752 inhibit different *Bd*GPL isolates, and only a small proportion of bacteria show broad scale inhibition
753 across the genetic variation exhibited by *Bd*GPL (Antwis et al., 2015). This, coupled with the
754 variation in host response to different isolates of *Bd*GPL (Farrer et al., 2011), means that potential
755 probiotics will need to account for differing virulence of *Bd* or that probiotics that show broad scale
756 inhibition must be identified and used.
757
758 In addition to the influence of environmental microbes and pathogens, the ecological interactions
759 among skin microbes would be expected to play a relevant role in structuring the skin microbiota.
760 Bacteria engage in the full breadth of ecological interactions, from antagonistic to facilitative
761 (reviewed by Faust & Raes, 2012). Many of these bacterial interactions are chemically mediated by
762 secondary metabolites, which can contribute to mutualistic interactions, such as cross-feeding or

763 syntrophy, in which two species benefit from each other's metabolic products (Woyke et al., 2006;
764 Faust & Raes, 2012; Loudon et al., 2014b). Secondary metabolite production is also influenced by
765 the composition of the bacterial community (Onaka et al., 2011), and therefore changes in the
766 community composition of the host (for example through environmental variation or diet) may
767 intrinsically lead to changes in the secondary metabolite profile of the total community. In addition to
768 mutualistic interactions, competition is common among microbes, and can occur via antibiotic
769 production (Kelsic et al., 2015). Other forms of competition can range from occupying space and
770 therefore inhibiting attachment of colonizing species, to more efficient consumption of shared
771 resources.
772
773 **C. Abiotic factors**
774
775 The skin microbiome in vertebrates is highly sensitive to changes in humidity and temperature (Grice
776 & Segre, 2011; Kueneman et al., 2014). Therefore, the skin microbial community structure might be
777 modified by exposure of the skin to different microclimates. This is particularly relevant for
778 ectotherms like amphibians, in which habitat-mediated thermoregulation can expose the host (and its
779 microbial symbionts) to a wide variety of microclimatic conditions over very short time periods
780 (Huey, 1991). Moreover, seasonal variation may influence host behavior by increasing host body
781 temperature (Rowley & Alford, 2013) and this could in turn modify the skin microbial structure.
782 Warmer temperatures can increase the skin sloughing frequency of anurans, thus reducing the
783 abundance of bacteria on the skin through frequent disturbance (Meyer et al., 2012; Ohmer et al.,
784 2014). In addition, thermal conditions influence the activity and production of antifungal metabolites
785 by symbiotic microbes on amphibian skin (Woodhams et al., 2014). For example, high temperatures
786 can limit the production of antimicrobial metabolites, such as violacein and prodigiosin produced by
787 *J. lividum* strains (Schloss et al., 2010; Woodhams et al. 2014). However, for other bacterial
788 probiotics, cooler temperatures may limit the production of antimicrobial products (Daskin et al.,
789 2014). The combined influences of environmental variation on microbiome stability are poorly
790 understood, and they likely vary among species from different habitats and ecosystems.
791
792 Moreover, temperature might also impact the skin microbiome by altering the interaction between the
793 amphibian immune system and invading pathogens. Immunity in ectotherms is strongly affected by
794 temperature (Raffel et al., 2006; Rollins-Smith et al., 2011; Rollins-Smith & Woodhams, 2012). In
795 general, low temperatures (4-10°C) are predicted to favor *Bd* (Woodhams et al., 2008; Voyles et al.,
796 2012), and under these conditions amphibian immune defenses are delayed or diminished (Rollins-
797 Smith et al., 2011; Rollins-Smith & Woodhams, 2012). In contrast, higher temperatures (25-30°C),
798 nearer to the maximum for *Bd* survival (Piotrowski et al., 2004; Stevenson et al., 2013), are predicted
799 to favor the amphibian host, enabling them to develop a more effective immune response (Rowley &
800 Alford, 2013). Similarly, *Bsal* infections can be cleared by host exposure to 25°C for 10 days (Blooi
801 et al., 2015). Thus, thermal preference of the host is associated with lower probabilities for *Bd* or
802 *Bsal* infection (Rowley & Alford, 2013). In this respect, the skin microbiome may also be affected by
803 pathogen invasions based on the host's and the pathogen's thermal preferences.
804
805 **Conflict of interest statement**
806 All authors declare that there are no conflicts of interests.
807
808 **Author contributions**
809 RH and ER contributed the original idea and outline. ER, RA, LB, MHB, MCB, RB, XH, AL, DM,
810 KM, LR, JW, DW and RH contributed the initial writing of specific sections. ER, LB, MHB, MH,
811 JK, VM, LR, SW, DW and RH contributed additional relevant ideas and sections as well as

812 structuring the manuscript. ER integrated all sections and produced all drafts of the manuscript,
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820

821 **Figure legends**

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

827 **Figure 2.** Data showing proof of concept of a network analysis to identify correlations among
828 bacterial and fungal OTUs on amphibian hosts. Network analyses depicting significantly correlated
829 bacterial and fungal OTUs (SparCC $r > 0.35$). All square nodes represent OTUs (either bacteria or
830 fungi).Red lines indicate negative correlations between two OTUs. Turquoise lines indicate positive
831 correlations between two OTUs. A) Assessing directionality of interactions found between all
832 bacteria and fungal taxa. Yellow = Betaproteobacteria, purple = Actinobacteria, blue = Fungi, white
833 = other bacterial OTUs. B) Assessing directionality of interactions found between all bacterial
834 interactions and Pathogen *Bd*. Yellow = bacteria that inhibit *Bd* in co-culture, blue = Unknown
835 interaction with *Bd* in co-culture. Center of network = fungal pathogen *Bd*.
836

837 **Figure 3.** Flow diagram indicating the steps of the probiotic filtering protocol proposed by Bletz et
838 al. (2013) that can be improved by omics data and integrative multi-omics analyses at different
839 stages: (A) Section 5.1: Integrated multi-omics methods can inform the isolation probiotic
840 candidates, (B) Section 5.2: probiotic candidates can be tested individually or in combination based
841 on omics results and (C) Section 5.3: omics approaches can track the effect of probiotic bacteria on
842 the host and on the environment. (D) Section 5.4: omics can provide probiotic candidates that can be
843 tested in mucosome assays.
844

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Commented [EA3]: I still need to add the new references

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