



University of  
**Salford**  
MANCHESTER

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

Rebollar, EA, Antwis, RE, Becker, MH, Belden, LK, Bletz, MC, Brucker, RM, Harrison, XA, Hughey, MC, Kueneman, JG, Loudon, AH, McKenzie, V, Medina, D, Minbiole, KPC, Rollins-Smith, LA, Walke, JB, Weiss, S, Woodhams, DC and Harris, RN

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

<b>Title</b>	Using “omics” and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases
<b>Authors</b>	Rebollar, EA, Antwis, RE, Becker, MH, Belden, LK, Bletz, MC, Brucker, RM, Harrison, XA, Hughey, MC, Kueneman, JG, Loudon, AH, McKenzie, V, Medina, D, Minbiole, KPC, Rollins-Smith, LA, Walke, JB, Weiss, S, Woodhams, DC and Harris, RN
<b>Type</b>	Article
<b>URL</b>	This version is available at: <a href="http://usir.salford.ac.uk/id/eprint/38077/">http://usir.salford.ac.uk/id/eprint/38077/</a>
<b>Published Date</b>	2016

USIR is a digital collection of the research output of the University of Salford. Where copyright permits, full text material held in the repository is made freely available online and can be read, downloaded and copied for non-commercial private study or research purposes. Please check the manuscript for any further copyright restrictions.

For more information, including our policy and submission procedure, please contact the Repository Team at: [usir@salford.ac.uk](mailto:usir@salford.ac.uk).

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

Eria A Rebollar<sup>1\*</sup>, Rachael E Antwis<sup>2,3,4</sup>, Matthew H Becker<sup>5</sup>, Lisa K Belden<sup>6</sup>, Molly C Bletz<sup>7</sup>, Robert M Brucker<sup>8</sup>, Xavier A Harrison<sup>3</sup>, Myra C Hughey<sup>6</sup>, Jordan G Kueneman<sup>9</sup>, Andrew H Loudon<sup>10</sup>, Valerie McKenzie<sup>9</sup>, Daniel Medina<sup>6</sup>, Kevin PC Minbiole<sup>11</sup>, Louise A Rollins-Smith<sup>12</sup>, Jennifer B Walke<sup>6</sup>, Sophie Weiss<sup>13</sup>, Douglas C Woodhams<sup>14</sup>, Reid N Harris<sup>1</sup>

1. Department of Biology, James Madison University, Harrisonburg, VA, USA.
2. Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa.
3. Institute of Zoology, Zoological Society of London, Regent’s Park, London, UK.
4. [School of Environment and Life Sciences, University of Salford, Salford, UK M5 4WT.](#)
5. Center for Conservation and Evolutionary Genetics, Smithsonian Conservation Biology Institute, National Zoological Park, Washington DC, USA
6. Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA.
7. Technische Universität Braunschweig, Zoological Institute, Braunschweig, Germany.
8. Rowland Institute At Harvard University, Cambridge, MA, USA.
9. Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO USA.
10. Department of Zoology and Biodiversity Research Centre, University of British Columbia, Vancouver, Canada.
11. Department of Chemistry, Villanova University, Villanova, PA, USA.
12. Departments of Pathology, Microbiology and Immunology and of Pediatrics, Vanderbilt University School of Medicine; Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA.
13. Department of Chemical and Biological Engineering, University of Colorado at Boulder, Boulder, CO USA.
14. Department of Biology, University of Massachusetts Boston, Boston MA, USA.

## \* Correspondence:

Dr. Eria A. Rebollar  
James Madison University,  
Biology Department,  
951 Carrier Drive MSC 7801,  
Harrisonburg, VA, USA  
[ea.rebollar@gmail.com](mailto:ea.rebollar@gmail.com)

**Running title:** Using multi-omics for probiotic selection

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

**Number of words:** 8,819,095

**Number of figures:** 3

**Number of boxes:** 1

## Using multi-omics for probiotic selection

### 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; Schroppe, 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

### Using multi-omics for probiotic selection

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

106  
107 In an effort to improve the chances of a positive outcome from the use of amphibian probiotics, a  
108 protocol that filters out ineffective candidates has been proposed (Bletz et al., 2013). This method  
109 was designed to identify successful probiotics for disease mitigation and species survival based on  
110 culture-dependent data (Bletz et al., 2013). In addition to the Bletz et al. (2013) filtering protocol, a  
111 mucosome assay, which aims to measure the protective function of the skin mucus, has recently been  
112 developed and applied to test potential probiotics (Woodhams et al., 2014).

113  
114 As new technologies and methods are being developed, it is desirable to further improve the filtering  
115 protocol with additional methods that can be used to facilitate probiotic candidate selection to  
116 increase the likelihood of success. In particular, high-throughput molecular techniques, colloquially  
117 called “omics” methods, have greatly increased our ability to characterize the taxonomic and genetic  
118 structure of bacterial communities, to estimate their functional capabilities and to evaluate their  
119 responses to stressors or pathogens (Grice & Siegre, 2011; Fierer et al., 2012; Greenblum et al.,  
120 2012; Knief et al., 2012; Jorth et al., 2014). Some of the omics methods developed to date are gene  
121 amplicon sequencing, shotgun metagenomics, transcriptomics, proteomics and metabolomics.  
122 Several studies and [extensive](#) reviews on these high-throughput molecular methods can be found in  
123 the literature (Fiehn 2002; Dettmer et al., 2007; Caporaso et al., 2011; Stewart et al., 2011; [Altelaar et](#)  
124 [al., 2012](#); Caporaso et al., 2012; [McGettigan 2013](#); [Franzosa et al., 2014](#); [Gust et al., 2014](#); [Manor et](#)  
125 [al., 2014](#); [Franzosa et al., 2015](#); [Loman & Pallen 2015](#)). Moreover, integrated multi-omics, which we  
126 define as the integrative analysis of data obtained from multiple omic methods, has the potential to  
127 greatly advance our understanding of ecological interactions occurring in microbial communities  
128 (Borenstein, 2012; McHardy et al., 2013; Meng et al., 2014). In this review, we establish an omics  
129 and integrated multi-omics framework with the aim of increasing the chances of selecting effective  
130 probiotic bacteria and achieving a successful disease mitigation strategy against EIDs. While these  
131 principles are applicable to other biological systems, for example in humans (Sánchez et al., 2013;  
132 Buffie et al., 2015; Forster & Lawley et al., 2015), we focus on applying these principles to the  
133 amphibian system, emphasizing currently [accessible](#) omics methods [that have been explored in](#)  
134 [amphibians](#) such as 16S rRNA gene amplicon sequencing (hereafter 16S amplicon sequencing),  
135 shotgun metagenomics, transcriptomics and metabolomics. [However other omics methods such as](#)  
136 [proteomics could be relevant in future studies to understand the interactions between hosts,](#)  
137 [pathogens and host-associated microbial communities.](#)

138  
139 In this review, we will (1) provide relevant knowledge about the skin microbiome in amphibians; (2)  
140 proceed with a description of the omics and integrated multi-omics methods that have been or could  
141 be applied to the amphibian system; (3) describe how omics and integrated multi-omics approaches  
142 can be incorporated into a previously described filtering protocol to identify probiotic candidates  
143 (Bletz et al., 2013); (4) provide important considerations and future directions that are relevant to the

## Using multi-omics for probiotic selection

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.](#)

### 2. Ecology of the amphibian skin microbiome

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 The amphibian skin microbiome is determined by the microbiota's interactions with host-associated  
157 factors and with abiotic and biotic factors (Figure 1, Box 1). Host-associated factors include host  
158 genetic diversity and the adaptive and innate immune systems (Box 1A), in addition to host behavior,  
159 ecology and development. Biotic factors include ecological interactions between skin symbiotic  
160 microbes and the microbial composition of environmental reservoirs (Box 1B), and abiotic factors  
161 include environmental conditions such as temperature and humidity (Box 1C). Altogether, the factors  
162 that influence the skin microbiome of amphibians determine the chemical composition of the skin  
163 mucus and in turn help determine the degree of host susceptibility against pathogens (Searle et al.,  
164 2011; Woodhams et al., 2014). Box 1 summarizes the current state of knowledge on the drivers of the  
165 amphibian skin microbiome. We now focus on omics and integrative multi-omics methods and how  
166 they can be used to address knowledge gaps that are key to developing effective probiotic strategies.

### 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.

## Using multi-omics for probiotic selection

### 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

## 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 ~~can~~ 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

### Using multi-omics for probiotic selection

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

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

#### 296 3.4. Metabolomics

297  
298  
299 Metabolites are the chemical intermediates and final products of cellular processes, and system-wide  
300 attempts to document all chemical species present in a selected biological sample (i.e.,  
301 metabolomics) have been undertaken for over a decade (Fiehn 2002, Bouslimani et al., 2015). In  
302 amphibians, differences in skin metabolite profiles (representing the sum of host and microbially-  
303 produced metabolites) across species have been identified (Umile et al., 2014). Metabolomics could  
304 also be used to compare species, populations or individuals with varying susceptibility to pathogens  
305 like *Bd*. For example, metabolite profiles can be compared between naïve populations and  
306 populations that have survived an epidemic of *Bd*, and metabolites that appear among survivors can  
307 be identified. The bacteria that produce these metabolites can then be tested for their inhibitory  
308 properties and probiotic potential.

309  
310 A complementary approach is to experimentally expose amphibians to *Bd* and compare the  
311 metabolite profiles of survivors and non-survivors. Individuals of the salamander *Plethodon cinereus*  
312 that were exposed to *Bd* and survived had significantly higher concentrations of the metabolite  
313 violacein on their skins than did individuals that died (Becker et al., 2009). This metabolite is  
314 produced by several species of bacteria, most notably *Janthinobacterium lividum*, which lives on the  
315 skin of many amphibian species and to inhibit *Bd in vitro* (Harris et al., 2006). Moreover, use of *J.*  
316 *lividum* as a probiotic on *Rana muscosa* decreased morbidity when individuals were exposed to *Bd*  
317 (Harris et al., 2009), although extension to another host species, the Panamanian golden frog (*A.*  
318 *zeteki*), failed to provide similar protection (Becker et al., 2011).

319  
320 One challenge in metabolomics is that metabolites vary enormously in chemical structure and  
321 reactivity, making the use of a single analytical tool to create a “chemical master inventory” nearly  
322 impossible. High-resolution mass spectrometry, often coupled with a separation technique such as  
323 high-performance liquid chromatography (LCMS), has led to significant strides in this arena  
324 (Dettmer et al., 2007). Since LCMS does not automatically provide molecular structure, further  
325 analysis is required, which can involve comparison to molecular databases. Free-access compendia of  
326 metabolite data have been published as early as 2005, in the first metabolomics web database  
327 METLIN, as well as the subsequent Human Metabolome Database (HMDB; Wishart et al., 2007;  
328 2009; 2013). Another challenge arises from the sheer number of data points generated by such  
329 analyses, the visualization of which can be daunting, although multivariate statistical analyses and  
330 analytical methods have been presented to address this chemometric challenge (Sharaf et al., 1986;  
331 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 The linear discriminant analysis (LDA) effect size method, LEfSe, can also be an informative method  
357 (Segata et al., 2011). LEfSe can be used to compare among groups that are biologically relevant and  
358 determine which features (organisms, clades, OTUs, genes, or functions) are significantly different  
359 (Albanese et al., 2015, Clemente et al., 2015, Zeng et al., 2015). LEfSe determines the factors that  
360 most likely explain differences between classes by coupling standard tests for statistical significance  
361 (Kruskal Wallis and Wilcoxon non parametric tests) with additional discriminant tests that estimate  
362 the magnitude of the effect (LDA score).

363  
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.

## Using multi-omics for probiotic selection

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

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

445  
446 Metagenomics, metatranscriptomics and metabolomics are important tools to determine molecular  
447 pathways present in microbial ecosystems. One of the main goals is integrating these multiple  
448 massive data sets to distinguish community patterns associated with a specific function such as host  
449 disease resistance. Protection against *Bd* in amphibians is likely achieved by a combination of  
450 functional pathways present in the skin microbiome in concert with the host's immune system.  
451 Therefore, the integration of multiple high dimensional datasets using predictive computational  
452 approaches such as bioinformatic predictive tools, multi-omic correlations and *in silico* models are  
453 key to predict functional outcomes within the skin microbiome (Borenstein, 2012; Langille et al.,  
454 2013; McHardy et al., 2013; Meng et al., 2014). One approach termed Reverse Ecology, offers a  
455 promising way to use high-throughput genomic data to infer ecological interactions from complex  
456 biological systems (Levy and Borenstein, 2012; 2014). It involves predicting the metabolic capacity  
457 of a biological system (including symbiotic systems) based on metagenomic data through the use of  
458 graph-theory based algorithms and genome-scale metabolic networks (Borenstein et al., 2008;  
459 Borenstein & Feldman, 2009; Freilich et al., 2009; Levy & Borenstein, 2012, Manor et al., 2014). To  
460 date, the amphibian skin microbiome has mainly been described using culture-dependent techniques  
461 and 16S amplicon sequencing. The use of these additional techniques may greatly improve our  
462 understanding of this microbial system and could allow us to identify fundamental metabolic  
463 pathways and ecological networks associated with defense against pathogens like *Bd*. For example,  
464 multi-omic correlations of 16S amplicon sequencing and metabolomics (McHardy et al., 2013) may  
465 allow us to determine bacterial taxa and metabolites associated with *Bd* inhibition in *Bd*-tolerant  
466 species and in individuals exposed to *Bd* in experimental trials.

467  
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

## Using multi-omics for probiotic selection

474 produce one or more anti-*Bd* metabolites that are associated with survival in a *Bd*-positive  
475 environment would be excellent probiotic candidates for bioaugmentation in at-risk populations.  
476

### 477 5. Using omics and integrating multi-omics data to inform probiotic selection through a 478 filtering protocol

479 Bletz et al. (2013) recently outlined sampling strategies and screening protocols for identifying ideal  
480 probiotics for amphibians (Figure 3). The framework involves (1) collecting and culturing skin  
481 microbes from selected host species; (2) isolating all morphologically-distinct colonies into pure  
482 culture; (3) testing each isolate for its ability to inhibit *Bd in vitro* (Bell et al., 2013); (4) testing  
483 highly inhibitory isolates for their ability to colonize and persist on amphibian skin; (5) and for those  
484 isolates that persist testing their ability to protect the host against *Bd* infection in clinical trials in the  
485 laboratory, followed by field trials (Bletz et al., 2013). In the case of the skin of some amphibian  
486 species, the dominant members of the microbiota are readily cultured (Walke et al. 2015), whereas  
487 some rare but prevalent members identified by 16S amplicon sequencing have been difficult to  
488 isolate in culture (Loudon et al. 2014a). Specialized media may be necessary to target microbes  
489 identified by omics approaches [including not only bacteria but also fungi](#). [Even though most of the  
490 probiotic search in amphibians has focused on bacterial candidates, the filtering protocol proposed by  
491 Bletz et al., \(2013\) could be also used to target potential fungal probiotics](#). Omic datasets and the  
492 integration of multi-omic analyses can facilitate the selection of the probiotic candidates that progress  
493 through this sampling and screening protocol. Below we describe the steps of the filtering protocol  
494 (Bletz et al., 2013) and the mucosome assay (Woodhams et al., 2014) that can be improved by omics  
495 and the integration of multi-omic approaches (Figure 3).  
496

#### 497 5.1.- Using omics data to inform the isolation of probiotic candidates.

498 A probiotic approach typically requires culturing and isolation of microbial species in order to test  
499 their antifungal functions and use only those species with desired properties. Given the taxonomic  
500 diversity [of the bacterial component](#) of the amphibian skin microbiome (Kueneman et al., 2014;  
501 Loudon et al., 2014a, Walke et al., 2014; Becker et al., 2015a; [Kueneman et al., 2015](#)), it would be  
502 useful to reduce the number of [bacteria-microorganisms](#) that one is trying to isolate and test for  
503 inhibition. Omics methods can streamline the [bacterial](#) isolation process by identifying promising  
504 probiotic taxa, which can then be isolated using media and culture conditions that favor or enrich for  
505 specific bacterial [or fungal](#) groups (Watve et al., 2000; Connon & Giovannoni, 2002; Rappé et al.,  
506 2002; Zengler et al., 2002, Vartoukian et al., 2010).  
507  
508  
509

510 Through the integration of multi-omics data, [bacterial-microbial](#) community members that are  
511 associated with surviving amphibian populations in the field and in laboratory experiments can be  
512 identified. We suggest using several methods such as indicator species analysis, the K-S Measure,  
513 LEfSe and co-occurrence networks in parallel to identify probiotic candidates. The main goal of  
514 using several methods is to obtain a list of OTUs that are congruent among methods. OTUs  
515 suggested as probiotic candidates by these culture-independent methods can be matched to bacterial  
516 isolates in pure culture identified with 16S rRNA Sanger sequencing (Woodhams et al., 2015) or to  
517 bacterial strains whose whole genome has been sequenced. These isolates would then proceed to  
518 testing for inhibition against *Bd* using *in vitro* challenge assays (Bell et al., 2013) or mucosome  
519 assays (Woodhams et al., 2014).  
520

Commented [B1]: Insert references for this

## Using multi-omics for probiotic selection

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

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

## Using multi-omics for probiotic selection

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

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

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

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

### 6. Important considerations and future directions

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

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

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

609 ~~In addition to~~Based on previous research on the amphibian system, this review has mainly focused on  
610 bacterial probiotics. ~~However,~~ future research may benefit by considering the micro-eukaryotic and  
611 viral components of the skin community. Indeed, fungi are important components of mammalian and  
612 amphibian skin (Underhill et al., 2014; Kueneman et al., 2015), and viruses have been linked to  
613 dysbiosis in the oral cavity (Edlund et al., 2015). Several studies have examined the importance of  
614  
615

Commented [B2]: delete

## 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 been 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 prebiotics 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 prebiotics \(postbiotics, Patel & Denning, 2013\) 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 \(Kesarcodi-Watson et al., 2008; Ringø et al., 2010; Bhardwaj et al., 2014; Lakshmanan et al., 2014; Hoyt et al., 2015; Papadimitriou et al., 2015; Uyeno et al., 2015\)](#). 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

## Using multi-omics for probiotic selection

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 (Tennesen &  
690 Blouin, 2007; Tennesen 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 (Tennesen & Blouin, 2007). However, apparent gene duplications allow for gradual genetic changes  
693 that appear to be positively selected in response to pathogens (Tennesen & 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



### Using multi-omics for probiotic selection

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, Mulet 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* (Mulet 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 (*BdGPL*) that has been associated with mass mortalities and  
747 rapid population declines of amphibians (Farrer et al., 2011; 2013). Within the *BdGPL* 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 *BdGPL* isolates, and only a small proportion of bacteria show broad scale inhibition  
753 across the genetic variation exhibited by *BdGPL* (Antwis et al., 2015). This, coupled with the  
754 variation in host response to different isolates of *BdGPL* (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

## Using multi-omics for probiotic selection

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 (Bloo  
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

## Using multi-omics for probiotic selection

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

814

### 815 Funding

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

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

### 845 REFERENCES

846

847 Aitchison J. (1986). The statistical analysis of compositional data, monographs on statistics and  
848 applied probability (Chapman & Hall Ltd, London) Reprinted (2003) with additional material by The  
849 Blackburn Press, Caldwell, NJ.

850 Akhter, N., Wu, B., Memon, A. M., and Mohsin, M. (2015). Probiotics and prebiotics associated with  
851 aquaculture: A review. *Fish Shellfish Immunol.* 45, 733–741. doi:10.1016/j.fsi.2015.05.038.

852 Albanese, D., De Filippo, C., Cavalieri, D., and Donati, C. (2015). Explaining diversity in  
853 metagenomic datasets by phylogenetic-based feature weighting. *PLOS Comput. Biol.* 11, e1004186.  
854 doi:10.1371/journal.pcbi.1004186.

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

### Using multi-omics for probiotic selection

- 855 Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome*  
856 *Biol.* 11, R106. doi:10.1186/gb-2010-11-10-r106.
- 857 Antwis, R. E., Preziosi, R. F., Harrison, X. A., and Garner, T. W. J. (2015). Amphibian symbiotic  
858 bacteria do not show universal ability to inhibit growth of the global pandemic lineage of  
859 *Batrachochytrium dendrobatidis*. *Appl. Environ. Microbiol.*, doi:10.1128/AEM.00010-15.
- 860 Arumugam, M., Raes, J., Pelletier, E., Paslier, D. Le, Batto, J., Bertalan, M., et al. (2013).  
861 Enterotypes of the human gut microbiome. *Nature* 473, 174–180. doi:10.1038/nature09944.
- 862 Barberán, A., Bates, S. T., Casamayor, E. O., and Fierer, N. (2012a). Using network analysis to  
863 explore co-occurrence patterns in soil microbial communities. *ISME J.* 6, 343–351.  
864 doi:10.1038/ismej.2011.119.
- 865 Barberán, A., Fernández-Guerra, A., Bohannan, B. J. M., and Casamayor, E. O. (2012b). Exploration  
866 of community traits as ecological markers in microbial metagenomes. *Mol. Ecol.* 21, 1909–1917.  
867 doi:10.1111/j.1365-294X.2011.05383.x.
- 868 Bataille, A., Fong, J. J., Cha, M., Wogan, G. O. U., Baek, H. J., Lee, H., et al. (2013). Genetic  
869 evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid  
870 fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Mol. Ecol.* 22, 4196–209.  
871 doi:10.1111/mec.12385.
- 872 Bataille, A., Cashins, S. D., Grogan, L., Skerratt, L. F., Hunter, D., Mcfadden, M., et al. (2015).  
873 Susceptibility of amphibians to chytridiomycosis is associated with MHC class II conformation.  
874 *Proc. R. Soc. B Biol. Sci.* doi:http://dx.doi.org/10.1098/rspb.2014.3127.
- 875 Becker, M. H., Brucker, R. M., Schwantes, C. R., Harris, R. N., and Minbiole, K. P. C. (2009). The  
876 bacterially produced metabolite violacein is associated with survival of amphibians infected with a  
877 lethal fungus. *Appl. Environ. Microbiol.* 75, 6635–6638. doi:10.1128/AEM.01294-09.
- 878 Becker, M. H., Harris, R. N., Minbiole, K. P. C., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L.  
879 K., et al. (2011). Towards a better understanding of the use of probiotics for preventing  
880 chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi:10.1007/s10393-012-0743-  
881 0.
- 882 Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015a).  
883 Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a  
884 lethal fungus. *Proc. R. Soc. B* 282, 20142881. doi:http://dx.doi.org/10.1098/rspb.2014.2881.
- 885 Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al.  
886 (2015b). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit  
887 growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641.  
888 doi:10.1111/mec.13135.
- 889 Bell, S. C., Alford, R. A., Garland, S., Padilla, G., and Thomas, A. D. (2013). Screening bacterial  
890 metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric  
891 assay. *Dis. Aquat. Organ.* 103, 77–85. doi:10.3354/dao02560.

### Using multi-omics for probiotic selection

- 892 Beman, J. M., Steele, J. A., and Fuhrman, J. A. (2011). Co-occurrence patterns for abundant marine  
893 archaeal and bacterial lineages in the deep chlorophyll maximum of coastal California. *ISME J.* 5,  
894 1077–1085. doi:10.1038/ismej.2010.204.
- 895 Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998).  
896 Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests  
897 of Australia and Central America. *Proc. Natl. Acad. Sci. U. S. A.* 95, 9031–9036.  
898 doi:10.1073/pnas.95.15.9031.
- 899 Blehert, D. S., Hicks, A. C., Behr, M., Meteyer, C. U., Berlowiskizier, B. M., Buckles, E. L., et al.  
900 (2009). Bat White-Nose Syndrome: An emerging pathogen? *Science.* 323:227.
- 901 Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., et al.  
902 (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective  
903 probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi:10.1111/ele.12099.
- 904 Bletz M. C., Rosa G. M., Crottini A., Courtois E. A., Schmeller D. S., Rabibisoa N. E. A. et al.  
905 (2015). Widespread presence of the pathogenic fungus *Batrachochytrium dendrobatidis* in wild  
906 amphibian communities in Madagascar. *Nat. Commun.*, 1–10. doi:10.1038/srep08633.
- 907 Blooi, M., Martel, A., Haesebrouck, F., Vercammen, F., Bonte, D., and Pasmans, F. (2015).  
908 Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium*  
909 *salamandrivorans*. *Sci. Rep.* 4, 35–38. doi:10.1038/srep08037.
- 910 Bolnick, D. I., Snowberg, L. K., Caporaso, J. G., Lauber, C., Knight, R., and Stutz, W. E. (2014).  
911 Major Histocompatibility Complex class IIb polymorphism influences gut microbiota composition  
912 and diversity. *Mol. Ecol.* doi:10.1111/mec.12846.
- 913 Borenstein, E., Kupiec, M., Feldman, M. W., and Ruppin, E. (2008). Large-scale reconstruction and  
914 phylogenetic analysis of metabolic environments. *Proc. Natl. Acad. Sci. U. S. A.* 105, 14482–14487.  
915 doi:10.1073/pnas.0806162105.
- 916 Borenstein, E., and Feldman, M. W. (2009). Topological signatures of species interactions in  
917 metabolic networks. *J. Comput. Biol.* 16, 191–200. doi:10.1089/cmb.2008.06TT.
- 918 Borenstein, E. (2012). Computational systems biology and in silico modeling of the human  
919 microbiome. *Brief. Bioinform.* 13, 769–780. doi:10.1093/bib/bbs022.
- 920 Bouslimani, A., Porto, C., Rath, C. M., Wang, M., Guo, Y., Gonzalez, A., et al. (2015). Molecular  
921 cartography of the human skin surface in 3D. *Proc. Natl. Acad. Sci.*, E2120-E2129.  
922 doi:10.1073/pnas.1424409112.
- 923 Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., et al.  
924 (2008). Amphibian chemical defense: antifungal metabolites of the microsymbiont  
925 *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–9.  
926 doi:10.1007/s10886-008-9555-7.

### Using multi-omics for probiotic selection

- 927 Brucker, R. M., and Bordenstein, S. R. (2012). The roles of host evolutionary relationships (genus:  
928 *Nasonia*) and development in structuring microbial communities. *Evolution*. 66, 349–362.  
929 doi:10.1111/j.1558-5646.2011.01454.x.
- 930 Brucker, R. M., and Bordenstein, S. R. (2013). The hologenomic basis of speciation: gut bacteria  
931 cause hybrid lethality in the genus *Nasonia*. *Science*. 341, 667–669. doi:10.1126/science.1240659.
- 932 Buffie, C. G., Bucci, V., Stein, R. R., McKenney, P. T., Ling, L., Gobourne, A., et al. (2015).  
933 Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*.  
934 *Nature* 517, 205–208. doi:10.1038/nature13828.
- 935 Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., et al. (2011). Global  
936 patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci.*  
937 *U. S. A.* 108 Suppl, 4516–4522. doi:10.1073/pnas.1000080107.
- 938 Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012).  
939 Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms.  
940 *ISME J.* 6, 1621–1624. doi:10.1038/ismej.2012.8.
- 941 Chagnot, C., Zorgani, M. A., Astruc, T., and Desvaux, M. (2013). Proteinaceous determinants of  
942 surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion  
943 perspective. *Front. Microbiol.* 4, 303. doi:10.3389/fmicb.2013.00303.
- 944 Clemente, J. C., Pehrsson, E. C., Blaser, M. J., Sandhu, K., Gao, Z., Wang, B., et al. (2015). The  
945 microbiome of uncontacted Amerindians. *Sci. Adv.* 1, e1500183–e1500183.  
946 doi:10.1126/sciadv.1500183.
- 947 Colombo, B. M., Scalvenzi, T., Benlamara, S., and Pollet, N. (2015). Microbiota and mucosal  
948 immunity in amphibians. *Front. Immunol.* 6, 1–15. doi:10.3389/fimmu.2015.00111.
- 949 Conlon, J. M. (2011). Structural diversity and species distribution of host-defense peptides in frog  
950 skin secretions. *Cell. Mol. Life Sci.* 68, 2303–2315. doi:10.1007/s00018-011-0720-8.
- 951 Cannon, S. A., and Giovannoni, S. J. (2002). High-throughput methods for culturing microorganisms  
952 in very-low-nutrient media yield diverse new marine isolates. *Appl. Environ. Microbiol.* 68, 3878–  
953 3885. doi:10.1128/AEM.68.8.3878.
- 954 Dale, C., and Moran, N. A. (2006). Molecular interactions between bacterial symbionts and their  
955 hosts. *Cell* 126, 453–465. doi:10.1016/j.cell.2006.07.014.
- 956 Daskin, J. H., Bell, S. C., Schwarzkopf, L., and Alford, R. A. (2014). Cool temperatures reduce  
957 antifungal activity of symbiotic bacteria of threatened amphibians - Implications for disease  
958 management and patterns of decline. *PLoS One* 9. doi:10.1371/journal.pone.0100378.
- 959 Daum, J. M., Davis, L. R., Bigler, L., and Woodhams, D. C. (2012). Hybrid advantage in skin  
960 peptide immune defenses of water frogs (*Pelophylax esculentus*) at risk from emerging pathogens.  
961 *Infect. Genet. Evol.* 12, 1854–1864. doi:10.1016/j.meegid.2012.07.024.

### Using multi-omics for probiotic selection

- 962 Deng, Y., Jiang, Y-H., Yang, Y., He, Z., Luo, F., and Zhou, J. (2012). Molecular ecological network  
963 analyses. *BMC Bioinformatics*. 13, 113. doi:10.1186/1471-2105-13-113.
- 964 Dettmer, K., Aronov, P. A., and Hammock, B. D. (2007). Mass spectrometry-based metabolomics.  
965 *Mass Spectrom. Rev.* 26, 51–78. doi:10.1002/mas.20108.
- 966 Ditzler, G., and Rosen, G. (2014). Feature subset selection for inferring relative importance of  
967 taxonomy. *ACM-BCB*, 673–679.
- 968 Dufrene, M., and Legendre, P. (1997). Species assemblages and indicator species: the need for a  
969 flexible asymmetrical approach. *ecol. Monogr.* 67, 345–366.
- 970 Edlund, A., Santiago-Rodriguez, T. M., Boehm, T. K., and Pride, D. T. (2015). Bacteriophage and  
971 their potential roles in the human oral cavity. *J. Oral Microbiol.* 7, 1–12.  
972 <http://www.sciencedirect.com/science/article/pii/B9780702028236500890>.
- 973 Ellison, A. R., Tunstall, T., DiRenzo, G. V., Hughey, M. C., Rebollar, E. A., Belden, L. K., Harris, R.  
974 N., Ibanez, R., Lips, K. R., and Zamudio, K. R. (2014). More than skin deep: functional genomic  
975 basis for resistance to amphibian chytridiomycosis. *Genome Biol. Evol.* 7, 286–298.  
976 doi:10.1093/gbe/evu285.
- 977 Farrer, R. A., Weinert, L., Bielby, J., Garner, T. W. J., Balloux, F., Clare, F., et al. (2011). Multiple  
978 emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent  
979 recombinant lineage. *Proc. Natl. Acad. Sci. U. S. A.* 108, 18732–18736. doi:  
980 10.1073/pnas.1111915108.
- 981 Farrer, R. A., Henk, D. A., Garner, T. W. J., Balloux, F., Woodhams, D. C., and Fisher, M. C.  
982 (2013). Chromosomal copy number variation, selection and uneven rates of recombination reveal  
983 cryptic genome diversity linked to pathogenicity. *PLoS Genet.* 9, e1003703.  
984 doi:10.1371/journal.pgen.1003703.
- 985 Faust, K., and Raes, J. (2012). Microbial interactions: from networks to models. *Nat. Rev. Microbiol.*  
986 10, 538–550. doi:10.1038/nrmicro2832.
- 987 Faust, K., Sathirapongsasuti, J. F., Izard, J., Segata, N., Gevers, D., Raes, J., et al. (2012). Microbial  
988 co-occurrence relationships in the Human Microbiome. *PLoS Comput. Biol.* 8.  
989 doi:10.1371/journal.pcbi.1002606.
- 990 Fiehn, O. (2002). Metabolomics - The link between genotypes and phenotypes. *Plant Mol. Biol.* 48,  
991 155–171. doi:10.1023/A:1013713905833.
- 992 Fierer, N., Ferrenberg, S., Flores, G. E., González, A., Kueneman, J., Legg, T., et al. (2012). From  
993 Animalcules to an Ecosystem: Application of Ecological Concepts to the Human Microbiome. *Annu.*  
994 *Rev. Ecol. Evol. Syst.* 43, 137–55. doi:10.1146/annurev-ecolsys-110411-160307.
- 995 Fisher, M. C., Garner, T. W. J., and Walker, S. F. (2009). Global emergence of *Batrachochytrium*  
996 *dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annu. Rev. Microbiol.* 63,  
997 291–310. doi:10.1146/annurev.micro.091208.073435.

### Using multi-omics for probiotic selection

- 998 Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., et al.  
999 (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*. 484, 186–94.  
1000 doi:10.1038/nature10947.
- 1001 Forster, S. C., and Lawley, T. D. (2015). Systematic discovery of probiotics. *Nat. Biotechnol.* 33, 47–  
1002 49. doi:10.1038/nbt.3111.
- 1003 Franzenburg, S., Walter, J., Künzel, S., Wang, J., Baines, J. F., Bosch, T. C. G., et al. (2013). Distinct  
1004 antimicrobial peptide expression determines host species-specific bacterial associations. *Proc. Natl.*  
1005 *Acad. Sci. U. S. A.* 110, E3730–8. doi:10.1073/pnas.1304960110.
- 1006 Franzosa, E. A., Morgan, X. C., Segata, N., Waldron, L., Reyes, J., Earl, A. M., et al. (2014).  
1007 Relating the metatranscriptome and metagenome of the human gut. *Proc. Natl. Acad. Sci. U. S. A.*  
1008 111, E2329–38. doi:10.1073/pnas.1319284111.
- 1009 Fraune, S., Anton-Erxleben, F., Augustin, R., Franzenburg, S., Knop, M., Schröder, K., et al. (2014).  
1010 Bacteria–bacteria interactions within the microbiota of the ancestral metazoan Hydra contribute to  
1011 fungal resistance. *ISME J.*, 1–14. doi:10.1038/ismej.2014.239.
- 1012 Freilich, S., Kreimer, A., Borenstein, E., Yosef, N., Sharan, R., Gophna, U., et al. (2009). Metabolic-  
1013 network-driven analysis of bacterial ecological strategies. *Genome Biol.* 10, R61. doi:10.1186/gb-  
1014 2009-10-6-r61.
- 1015 Friedman, J., and Alm, E. J. (2012). Inferring correlation networks from genomic survey data. *PLoS*  
1016 *Comput. Biol.* 8, 1–11. doi:10.1371/journal.pcbi.1002687.
- 1017 Giannoukos, G., Ciulla, D. M., Huang, K., Haas, B. J., Izard, J., Levin, J. Z., et al. (2012). Efficient  
1018 and robust RNA-seq process for cultured bacteria and complex community transcriptomes. *Genome*  
1019 *Biol.* 13, R23. doi:10.1186/gb-2012-13-3-r23.
- 1020 Greenblum, S., Turnbaugh, P. J., and Borenstein, E. (2012). Metagenomic systems biology of the  
1021 human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel  
1022 disease. *Proc. Natl. Acad. Sci.* 109, 594–599. doi:10.1073/pnas.1116053109.
- 1023 Grice, E. A., and Segre, J. A. (2011). The skin microbiome. *Nat rev Microbiol.* 9, 244–253.  
1024 doi:10.1038/nrmicro2537.
- 1025 Grzymiski, J. J., Murray, A. E., Campbell, B. J., Kaplarevic, M., Gao, G. R., Lee, C., et al. (2008).  
1026 Metagenome analysis of an extreme microbial symbiosis reveals eurythermal adaptation and  
1027 metabolic flexibility. *Proc. Natl. Acad. Sci. U. S. A.* 105, 17516–17521.  
1028 doi:10.1073/pnas.0802782105.
- 1029 Gust, K. A., Najjar, F. Z., Habib, T., Lotufo, G. R., Piggot, A. M., Fouke, B. W., et al. (2014). Coral-  
1030 zooxanthellae meta-transcriptomics reveals integrated response to pollutant stress. *BMC Genomics*  
1031 15, 591. doi:10.1186/1471-2164-15-591.



### Using multi-omics for probiotic selection

- 1032 Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen  
1033 *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species.  
1034 *Ecohealth* 3, 53–56. doi:10.1007/s10393-005-0009-1.
- 1035 Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al.  
1036 (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME*  
1037 *J.* 3, 818–824. doi:10.1038/ismej.2009.27.
- 1038 Hawkes, C. V., and Keitt, T. H. (2015). Resilience vs. historical contingency in microbial responses  
1039 to environmental change. *Ecol. Lett.* 18, 612–625. doi:10.1111/ele.12451.
- 1040 Holden, W. M., Hanlon, S. M., Woodhams, D. C., Chappell, T. M., Wells, H. L., Glisson, S. M., et  
1041 al. (2015). Skin bacteria provide early protection for newly metamorphosed southern leopard frogs  
1042 (*Rana sphenocephala*) against the frog-killing fungus, *Batrachochytrium dendrobatidis*. *Biol.*  
1043 *Conserv.* 187, 91–102. doi:10.1016/j.biocon.2015.04.007.
- 1044 Hoyt, J. R., Cheng, T. L., Langwig, K. E., Hee, M. M., Frick, W. F., and Kilpatrick, A. M. (2015).  
1045 Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent  
1046 of white-nose syndrome. *PLoS One* 10, e0121329. doi:10.1371/journal.pone.0121329.
- 1047 Huey, R. B. (1991). Physiological consequences of habitat selection. *American Naturalist*, S91-S115.
- 1048 Imangaliyev, S., Keijsers, B., Crielaard, W., and Tsivtsivadze, E. (2015). Personalized microbial  
1049 network inference via co-regularized spectral clustering. *Methods* 83, 28–35.  
1050 doi:10.1016/j.ymeth.2015.03.017.
- 1051 Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog  
1052 skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci.* 111,  
1053 E5049–E5058. doi:10.1073/pnas.1412752111.
- 1054 Jorth, P., Turner, K. H., Gumus, P., Nizam, N., Buduneli, N., and Whiteley, M. (2014).  
1055 Metatranscriptomics of the human oral microbiome during health and disease. *MBio* 5, 1–10.  
1056 doi:10.1128/mBio.01012-14.
- 1057 Katzenback, B. A., Holden, H. A., Falardeau, J., Childers, C., Hadj-Moussa, H., Avis, T. J., et al.  
1058 (2014). Regulation of the *Rana sylvatica* brevinin-1SY antimicrobial peptide during development and  
1059 in dorsal and ventral skin in response to freezing, anoxia and dehydration. *J. Exp. Biol.* 217, 1392–  
1060 401. doi:10.1242/jeb.092288.
- 1061 Kelsic, E. D., Zhao, J., Vetsigian, K., and Kishony, R. (2015). Counteraction of antibiotic production  
1062 and degradation stabilizes microbial communities. *Nature* 521, 516–519.  
1063 <http://dx.doi.org/10.1038/nature14485>.
- 1064 Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., and Gibson, L. (2008). Probiotics in aquaculture:  
1065 The need, principles and mechanisms of action and screening processes. *Aquaculture* 274, 1–14.  
1066 doi:10.1016/j.aquaculture.2007.11.019.

### Using multi-omics for probiotic selection

- 1067 Klemm, P., and Schembri, M. A. (2000). Bacterial adhesins: function and structure. *Int. J. Med.*  
1068 *Microbiol.* 290, 27–35. doi:10.1016/S1438-4221(00)80102-2.
- 1069 Kline, K. A., Fälker, S., Dahlberg, S., Normark, S., and Henriques-Normark, B. (2009). Bacterial  
1070 adhesins in host-microbe interactions. *Cell Host Microbe* 5, 580–592.  
1071 doi:10.1016/j.chom.2009.05.011.
- 1072 Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., et al. (2012).  
1073 Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice.  
1074 *ISME J.* 6, 1378–1390. doi:10.1038/ismej.2011.192.
- 1075 Knights, D., Costello, E. K., and Knight, R. (2011a). Supervised classification of human microbiota.  
1076 *FEMS Microbiol. Rev.* 35, 343–359. doi:10.1111/j.1574-6976.2010.00251.x.
- 1077 Knights, D., Kuczynski, J., Koren, O., Ley, R. E., Field, D., Knight, R., DeSantis, T. Z., and Kelley,  
1078 S. T. (2011b). Supervised classification of microbiota mitigates mislabeling errors. *ISME J.* 5, 570–  
1079 573. doi:10.1038/ismej.2010.148.
- 1080 Knights, D., Parfrey, L. W., Zaneveld, J., Lozupone, C., and Knight, R. (2011c). Human-associated  
1081 microbial signatures: Examining their predictive value. *Cell Host Microbe* 10, 292–296.  
1082 doi:10.1016/j.chom.2011.09.003.
- 1083 Kolmogorov, A. (1933). Sulla determinazione empirica di una legge di distribuzione. *Giornale*  
1084 *dell'Istituto Italiano degli Attuari* 4, 83–91.
- 1085 Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J.  
1086 (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol.*  
1087 *Ecol.* 23, 1238–1250. doi:10.1111/mec.12510.
- 1088 Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and V. J.  
1089 McKenzie. Inhibitory bacteria are associated with reduced fungi on early developmental life stages of  
1090 endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME Journal*. *In press*.
- 1091 Küng, D., Bigler, L., Davis, L. R., Gratwicke, B., Griffith, E., and Woodhams, D. C. (2014). Stability  
1092 of microbiota facilitated by host immune regulation: Informing probiotic strategies to manage  
1093 amphibian disease. *PLoS One* 9. doi:10.1371/journal.pone.0087101.
- 1094 Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A, et al.  
1095 (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene  
1096 sequences. *Nat. Biotechnol.* 31, 814–21. doi:10.1038/nbt.2676.
- 1097 Langwig, K. E., Voyles, J., Wilber, M. Q., Frick, W. F., Murray, K. A., Bolker, B. M., et al. (2015).  
1098 Context-dependent conservation responses to emerging wildlife diseases. *Front. Ecol. Environ.* 13,  
1099 195–202. doi:10.1890/140241.
- 1100 Lauer, A., Simon, M., Banning, J. L., Andre, E., Duncan, K., and Harris, R. N. (2007). Common  
1101 cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia*,  
1102 630–640.

Commented [B4]: Change

### Using multi-omics for probiotic selection

- 1103 Lawley, T. D., Clare, S., Walker, A. W., Stares, M. D., Connor, T. R., Raisen, C., et al. (2012).  
1104 Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves  
1105 relapsing *Clostridium difficile* disease in mice. *PLoS Pathog.* 8. doi:10.1371/journal.ppat.1002995.
- 1106 Levy, R., and Borenstein, E. (2012). Reverse Ecology: from systems to environments and back. *Evol.*  
1107 *Syst. Biol.* 751, 329–53. doi:10.1007/978-1-4614-3567-9.
- 1108 Levy, R., and Borenstein, E. (2013). Metabolic modeling of species interaction in the human  
1109 microbiome elucidates community-level assembly rules. *Proc. Natl. Acad. Sci. U. S. A.* 110, 12804–  
1110 9. doi:10.1073/pnas.1300926110.
- 1111 Levy, R., and Borenstein, and E. (2014). Metagenomic systems biology and metabolic modeling of  
1112 the human microbiome. From species composition to community assembly rules. *Gut Microbes* 5, 1–  
1113 6.
- 1114 Liao, H-L., Chen, Y., Bruns, T. D., Peay, K. G., Taylor, J. W., Branco, S., et al. (2014).  
1115 Metatranscriptomic analysis of ectomycorrhizal roots reveals genes associated with *Piloderma-Pinus*  
1116 symbiosis: improved methodologies for assessing gene expression in situ. *Environ. Microbiol.* 16,  
1117 3730–3742. doi:10.1111/1462-2920.12619.
- 1118 Loftus, S. C., House, L. L., Hughey, M. C., Walke, J. B., Becker, M. H., and Belden, L. K. (2015).  
1119 "Dimension reduction for multinomial models via a Kolmogorov-Smirnov Measure (KSM)".  
1120 (Blacksburg, Virginia: Department of Statistics, Virginia Tech).  
1121 [http://www.stat.vt.edu/research/Technical\\_Reports/TechReport15-1.pdf](http://www.stat.vt.edu/research/Technical_Reports/TechReport15-1.pdf).
- 1122 Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V., et al.  
1123 (2014a). Microbial community dynamics and effect of environmental microbial reservoirs on red-  
1124 backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–40. doi:10.1038/ismej.2013.200.
- 1125 Loudon, A., Holland, J., Umile, T., Burzynski, E., Minbiole, K. P. C., and Harris, R. N. (2014b).  
1126 Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal  
1127 metabolites. *Front. Microbiol.* 5, 1–8. doi:10.3389/fmicb.2014.00441.
- 1128 Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion  
1129 for RNA-Seq data with DESeq2. *Gen Biol.* 15:550, 1–21. doi:10.1101/002832.
- 1130 Lovell, D. M.W., Taylor, J., Zwart, A., Helliwell, C. (2010). Caution! compositions! can constraints  
1131 on omics data lead analyses astray? *CSIRO Report Number: EP10994*  
1132 [http://www.csiro.au/en/Organisation-Structure/Divisions/Computational-Informatics/Caution-](http://www.csiro.au/en/Organisation-Structure/Divisions/Computational-Informatics/Caution-Compositions.aspx)  
1133 [Compositions.aspx](http://www.csiro.au/en/Organisation-Structure/Divisions/Computational-Informatics/Caution-Compositions.aspx)
- 1134 Macfoy, C., Danosus, D., Sandit, R., Jones, T. H., Garraffo, H. M., Spande, T. F., et al. (2005).  
1135 Alkaloids of anuran skin: Antimicrobial function? *Zeitschrift fur Naturforsch. - Sect. C J. Biosci.* 60,  
1136 932–937.
- 1137 Manor, O., Levy, R., and Borenstein, E. (2014). Mapping the inner workings of the microbiome:  
1138 Genomic- and metagenomic-based study of metabolism and of metabolic interactions in the human  
1139 gut microbiome. *Cell Metab.* 20, 742–752. doi:10.1016/j.cmet.2014.07.021.

### Using multi-omics for probiotic selection

- 1140 Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., et al. (2013).  
1141 *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proc.*  
1142 *Natl. Acad. Sci. U. S. A.* 110, 15325–9. doi:10.1073/pnas.1307356110.
- 1143 Martel, A., Blooi, M., Fisher, M. C., Farrer, R. A., Schmidt, B. R., Tobler, U., et al. (2014). Recent  
1144 introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*. 346, 630–31.
- 1145 McHardy, I. H., Goudarzi, M., Tong, M., Ruegger, P. M., Schwager, E., Weger, J. R., et al. (2013).  
1146 Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface  
1147 reveals exquisite inter-relationships. *Microbiome*. 1, 17. doi:10.1186/2049-2618-1-17.
- 1148 McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting  
1149 amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–  
1150 596. doi:10.1038/ismej.2011.129.
- 1151 McMahon, T. A., Sears, B. F., Venesky, M. D., Bessler, S. M., Brown, J. M., Deutsch, K., et al.  
1152 (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal  
1153 immunosuppression. *Nature*. 511, 224–227. doi:10.1038/nature13491.
- 1154 McMurdie, P. J., and Holmes, S. (2014). Waste not, want not: why rarefying microbiome data is  
1155 inadmissible. *PLoS Comput. Biol.* 10. doi:10.1371/journal.pcbi.1003531.
- 1156 Meng, C., Kuster, B., Culhane, A. C., and Moghaddas Gholami, A. (2014). A multivariate approach  
1157 to the integration of multi-omics datasets. *BMC Bioinformatics* 15, 162. doi:10.1186/1471-2105-15-  
1158 162.
- 1159 Metzker, M. L. (2010). Sequencing technologies - the next generation. *Nat. Rev. Genet.* 11, 31–46.  
1160 <http://dx.doi.org/10.1038/nrg2626>.
- 1161 Meyer, E. A., Cramp, R. L., Bernal, M. H., and Franklin, C. E. (2012). Changes in cutaneous  
1162 microbial abundance with sloughing: Possible implications for infection and disease in amphibians.  
1163 *Dis. Aquat. Organ.* 101, 235–242. doi:10.3354/dao02523.
- 1164 Michaels, C. J., Antwis, R. E., and Preziosi, R. F. (2014). Impact of plant cover on fitness and  
1165 behavioural traits of captive red-eyed tree frogs (*Agalychnis callidryas*). *PLoS One* 9, e95207.  
1166 doi:10.1371/journal.pone.0095207.
- 1167 Muletz, C. R., Myers, J. M., Domangue, R. J., Herrick, J. B., and Harris, R. N. (2012). Soil  
1168 bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by  
1169 *Batrachochytrium dendrobatidis*. *Biol. Conserv.* 152, 119–126. doi:10.1016/j.biocon.2012.03.022.
- 1170 Myers, J. M., Ramsey, J. P., Blackman, A. L., Nichols, A. E., Minbiole, K. P. C., and Harris, R. N.  
1171 (2012). Synergistic inhibition of the lethal fungal pathogen *Batrachochytrium dendrobatidis*: the  
1172 combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog *Rana*  
1173 *muscosa*. *J. Chem. Ecol.* 38, 958–65. doi:10.1007/s10886-012-0170-2.

### Using multi-omics for probiotic selection

- 1174 Nakatsuji, T., Chiang, H-I., Jiang, S. B., Nagarajan, H., Zengler, K., and Gallo, R. L. (2013). The  
1175 microbiome extends to subepidermal compartments of normal skin. *Nat. Commun.* 4.  
1176 doi:10.1038/ncomms2441.
- 1177 Ohmer, M. E. B., Cramp, R. L., White, C. R., and Franklin, C. E. (2014). Skin sloughing rate  
1178 increases with chytrid fungus infection load in a susceptible amphibian. *Funct. Ecol.*  
1179 doi:10.1111/1365-2435.12370.
- 1180 Onaka, H., Mori, Y., Igarashi, Y., and Furumai, T. (2011). Mycolic acid-containing bacteria induce  
1181 natural-product biosynthesis in *Streptomyces* species. *Appl. Environ. Microbiol.* 77, 400–406.  
1182 doi:10.1128/AEM.01337-10.
- 1183 Papadimitriou, K., Zoumpopoulou, G., Foligné, B., Alexandraki, V., Kazou, M., Pot, B., et al.  
1184 (2015). Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches.  
1185 *Front. Microbiol.* 6, 1–28. doi:10.3389/fmicb.2015.00058.
- 1186 Parfrey, L. W., Walters, W. A., Lauber, C. L., Clemente, J. C., Berg-Lyons, D., Teiling, C., et al.  
1187 (2014). Communities of microbial eukaryotes in the mammalian gut within the context of  
1188 environmental eukaryotic diversity. *Front. Microbiol.* 5, 1–13. doi:10.3389/fmicb.2014.00298.
- 1189 Pask, J. D., Woodhams, D. C., and Rollins-Smith, L. A. (2012). The ebb and flow of antimicrobial  
1190 skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. *Glob. Chang.*  
1191 *Biol.* 18, 1231–1238. doi:10.1111/j.1365-2486.2011.02622.x.
- 1192 Patti, G. J., Tautenhahn, R., Rinehart, D., Cho, K., Shriver, L. P., Manchester, M., et al. (2013). A  
1193 view from above: Cloud plots to visualize global metabolomic data. *Anal. Chem.* 85, 798–804.  
1194 doi:10.1021/ac3029745.
- 1195 Pearson, K. (1909). Determination of the coefficient of correlation. *Science.* 30: 23-25.
- 1196 Piotrowski, J. S., Annis, S. L., and Longcore, J. E. (2004). Physiology of *Batrachochytrium*  
1197 *dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia.* 96, 9–15.  
1198 <http://www.ncbi.nlm.nih.gov/pubmed/21148822>.
- 1199 Price, S. J., Garner, T. W. J., Nichols, R. A., Balloux, F., Ayres, C., Mora-Cabello de Alba, A., et al.  
1200 (2014). Collapse of amphibian communities due to an introduced *Ranavirus*. *Curr. Biol.*, 2586–2591.  
1201 doi:10.1016/j.cub.2014.09.028.
- 1202 Price, S. J., Garner, T. W. J., Balloux, F., Ruis, C., Paszkiewicz, K. H., Moore, K., et al. (2015). A de  
1203 novo assembly of the common frog (*Rana temporaria*) transcriptome and comparison of transcription  
1204 following exposure to *Ranavirus* and *Batrachochytrium dendrobatidis*. *PLoS One* 10, e0130500.  
1205 doi:10.1371/journal.pone.0130500.
- 1206 R Core Team. (2014). R: A language and environment for statistical computing. R Foundation for  
1207 Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.

### Using multi-omics for probiotic selection

- 1208 Raffel, T. R., Rohr, J. R., Kiesecker, J. M., and Hudson, P. J. (2006). Negative effects of changing  
1209 temperature on amphibian immunity under field conditions. *Funct. Ecol.* 20, 819–828.  
1210 doi:10.1111/j.1365-2435.2006.01159.x.
- 1211 Ramsey, J. P., Reinert, L. K., Harper, L. K., Woodhams, D. C., and Rollins-Smith, L. A. (2010).  
1212 Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian  
1213 declines, in the South African clawed frog, *Xenopus laevis*. *Infect. Immun.* 78, 3981–92.  
1214 doi:10.1128/IAI.00402-10.
- 1215 Rappé, M. S., Connon, S. A., Vergin, K. L., and Giovannoni, S. J. (2002). Cultivation of the  
1216 ubiquitous SAR11 marine bacterioplankton clade. *Nature.* 418, 630–633. doi:10.1038/nature00917.
- 1217 Reid, G., Younes, J. A., Van der Mei, H. C., Gloor, G. B., Knight, R., and Busscher, H. J. (2011).  
1218 Microbiota restoration: natural and supplemented recovery of human microbial communities. *Nat.*  
1219 *Rev. Microbiol.* 9, 27–38. doi:10.1038/nrmicro2473.
- 1220 Reshef, D. N., Reshef, Y. A., Finucane, H. K., Grossman, S. R., Mcvean, G., Turnbaugh, P. J., et al.  
1221 (2011). Detecting novel associations in large data sets. *Science.* 334, 1518–1524.  
1222 doi:10.1126/science.1205438.
- 1223 Ringø, E., Olsen, R. E., Gifstad, T., Dalmo, R. A., Amlund, H., Hemre, G. I., et al. (2010). Prebiotics  
1224 in aquaculture: A review. *Aquac. Nutr.* 16, 117–136. doi:10.1111/j.1365-2095.2009.00731.x.
- 1225 Rizzetto, L., Filippo, C. De, and Cavalieri, D. (2015). Fungal genomics & biology mycobiota: micro-  
1226 eukaryotes inhabiting our body as commensals or opportunistic pathogens. *Fungal Genom. Biol.* 4,  
1227 1–9. doi:10.4172/2165-8056.1000120.
- 1228 Roberts, D. W. (2007). *laBdsv*: Ordination and multivariate analysis for ecology. *R package*  
1229 *version, 1(1)*.
- 1230 Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K., and Woodhams, D. C. (2011).  
1231 Amphibian immune defenses against chytridiomycosis: impacts of changing environments. *Integr.*  
1232 *Comp. Biol.* 51, 552–62. doi:10.1093/icb/icr095.
- 1233 Rollins-Smith, L. A., and Woodhams D. C. 2012. Amphibian immunity: staying in tune with the  
1234 environment. In: *Eco-Immunology*. Demas, G.E. and Nelson, R.J. eds. Oxford University Press,  
1235 Oxford, United Kingdom. pp. 92-143.
- 1236 Rosvall, M., and Bergstrom, C. T. (2010). Mapping change in large networks. *PLoS One* 5.  
1237 doi:10.1371/journal.pone.0008694.
- 1238 Rowley, J. J. L., and Alford, R. A. (2013). Hot bodies protect amphibians against chytrid infection in  
1239 nature. *Sci. Rep.* 3, 1515. doi:10.1038/srep01515.
- 1240 Ruan, Q., Dutta, D., Schwalbach, M. S., Steele, J. A., Fuhrman, J. A., and Sun, F. (2006). Local  
1241 similarity analysis reveals unique associations among marine bacterioplankton species and  
1242 environmental factors. *Bioinformatics.* 22, 2532–2538. doi:10.1093/bioinformatics/btl417.

### Using multi-omics for probiotic selection

- 1243 Sánchez, B., Ruiz, L., Gueimonde, M., and Margolles, A. (2013). Omics for the study of probiotic  
1244 microorganisms. *Food Res. Int.* 54, 1061–1071. doi:10.1016/j.foodres.2013.01.029.
- 1245 Sanders, J. G., Powell, S., Kronauer, D. J. C., Vasconcelos, H. L., Frederickson, M. E., and Pierce, N.  
1246 E. (2014). Stability and phylogenetic correlation in gut microbiota: lessons from ants and apes. *Mol.*  
1247 *Ecol.* 23, 1268–1283. doi:10.1111/mec.12611.
- 1248 Savage, A. E., and Zamudio, K. R. (2011). MHC genotypes associate with resistance to a frog-killing  
1249 fungus. *Proc. Natl. Acad. Sci. U. S. A.* 108, 16705–10. doi:10.1073/pnas.1106893108.
- 1250 Savage, A. E., Kiemnec-Tyburczy, K. M., Ellison, A. R., Fleischer, R. C., and Zamudio, K. R.  
1251 (2014). Conservation and divergence in the frog immunome: Pyrosequencing and de novo assembly  
1252 of immune tissue transcriptomes. *Gene* 542, 98–108. doi:10.1016/j.gene.2014.03.051.
- 1253 Schloegel, L. M., Toledo, L. F., Longcore, J. E., Greenspan, S. E., Vieira, C. A., Lee, M., et al.  
1254 (2012). Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the  
1255 bullfrog trade. *Mol. Ecol.* 21, 5162–5177. doi:10.1111/j.1365-294X.2012.05710.x.
- 1256 Schloss, P. D., Allen, H. K., Klimowicz, A. K., Mlot, C., Gross, J. A., Savengsuksa, S., et al. (2010).  
1257 Psychrotrophic strain of *Janthinobacterium lividum* from a cold Alaskan soil produces prodigiosin.  
1258 *DNA Cell Biol.* 29, 533–541. doi:10.1089/dna.2010.1020.
- 1259 Schrope, M. (2014). Sea star wasting. *Proc. Natl. Acad. Sci. U. S. A.* 111, 6855.  
1260 doi:10.1073/pnas.1404650111.
- 1261 Searle, C. L., Gervasi, S. S., Hua, J., Hammond, J. I., Relyea, R. A., Olson, D. H., et al. (2011).  
1262 Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen.  
1263 *Conserv. Biol.* 25, 965–74. doi:10.1111/j.1523-1739.2011.01708.x.
- 1264 Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011).  
1265 Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. doi:10.1186/gb-2011-12-  
1266 6-r60.
- 1267 Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape:  
1268 a software environment for integrated models of biomolecular interaction networks. *Genome Res.*  
1269 2498–2504. doi:10.1101/gr.1239303.
- 1270 Sharaf, M. A., Illman, D. L., Kowalski, B. R., eds. (1986). *Chemometrics*. New York: Wiley. ISBN  
1271 0471831069.
- 1272 Smirnov, N. (1936). Sur la distribution de  $\omega^2$  (criterium de M. R. v. Mises). *Comptes rendus de*  
1274 *l'Académie des Sciences*. 202, 449-452.
- 1275 Spearman, C. (1904). The proof and measurement of association between two things. *The American*  
1276 *journal of psychology*. 15: 72-101.
- 1277 Spor, A., Koren, O., and Ley, R. (2011). Unravelling the effects of the environment and host  
1278 genotype on the gut microbiome. *Nat. Rev. Microbiol.* 9, 279–290. doi:10.1038/nrmicro2540.

### Using multi-omics for probiotic selection

- 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*  
1281 1, 11. doi:10.1186/2049-2618-1-11.
- 1282 Steele, J. A., Countway, P. D., Xia, L., Vigil, P. D., Beman, J. M., Kim, D. Y., et al. (2011). Marine  
1283 bacterial, archaeal and protistan association networks reveal ecological linkages. *ISME J.* 5, 1414–  
1284 1425. doi:10.1038/ismej.2011.24.
- 1285 Stevenson, L. A., Alford, R. A., Bell, S. C., Roznik, E. A., Berger, L., and Pike, D. A. (2013).  
1286 Variation in thermal performance of a widespread pathogen, the amphibian chytrid fungus  
1287 *Batrachochytrium dendrobatidis*. *PLoS One* 8, 1–14. doi:10.1371/journal.pone.0073830.
- 1288 Stewart, F. J., Sharma, A. K., Bryant, J. A., Eppley, J. M., and DeLong, E. F. (2011). Community  
1289 transcriptomics reveals universal patterns of protein sequence conservation in natural microbial  
1290 communities. *Genome Biol.* 12, R26. doi:10.1186/gb-2011-12-3-r26.
- 1291 Stice, M. J., and Briggs, C. J. (2010). Immunization is ineffective at preventing infection and  
1292 mortality due to the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *J. Wildl. Dis.* 46, 70–  
1293 7. doi:10.7589/0090-3558-46.1.70.
- 1294 Susi, H., Barrès, B., Vale, P. F., and Laine, A-L. (2015). Co-infection alters population dynamics of  
1295 infectious disease. *Nat. Commun.* 6, 5975. doi:10.1038/ncomms6975.
- 1296 Tennessen, J. A., and Blouin, M. S. (2007). Selection for antimicrobial peptide diversity in frogs  
1297 leads to gene duplication and low allelic variation. *J. Mol. Evol.* 65, 605–615. doi:10.1007/s00239-  
1298 007-9045-5.
- 1299 Tennessen, J. A., Woodhams, D. C., Chaurand, P., Reinert, L. K., Billheimer, D., Shyr, Y., et al.  
1300 (2009). Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (*Rana*  
1301 *pipiens*) populations suggest intraspecies differences in resistance to pathogens. *Dev. Comp.*  
1302 *Immunol.* 33, 1247–1257. doi:10.1016/j.dci.2009.07.004.
- 1303 Umile, T. P., McLaughlin, P. J., Johnson, K. R., Honarvar, S., Blackman, A. L., Burzynski, E. A., et  
1304 al. (2014). Nonlethal amphibian skin swabbing of cutaneous natural products for HPLC  
1305 fingerprinting. *Anal. Methods.* 6, 3277. doi:10.1039/c4ay00566j.
- 1306 Underhill, D. M., and Iliev, I. D. (2014). The mycobiota: interactions between commensal fungi and  
1307 the host immune system. *Nat Rev Immunol.* 14, 405–416. <http://dx.doi.org/10.1038/nri3684>.
- 1308 Uyeno, Y., Shigemori, S., and Shimosato, T. (2015). Effect of probiotics/prebiotics on cattle health  
1309 and productivity. *Microbes Environ.* 30, 126–132. doi:10.1264/jsme2.ME14176.
- 1310 Vartoukian, S. R., Palmer, R. M., and Wade, W. G. (2010). Strategies for culture of “unculturable”  
1311 bacteria. *FEMS Microbiol. Lett.* 309, 1–7. doi:10.1111/j.1574-6968.2010.02000.x.
- 1312 Voyles, J., Johnson, L. R., Briggs, C. J., Cashins, S. D., Alford, R. A., Berger, L., et al. (2012).  
1313 Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal



### Using multi-omics for probiotic selection

- 1314 pathogen associated with the global loss of amphibians. *Ecol. Evol.* 2, 2241–2249.  
1315 doi:10.1002/ece3.334.
- 1316 Wake, D. B., and Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view  
1317 from the world of amphibians. *Proc. Natl. Acad. Sci. U. S. A.* 105, 11466–11473.  
1318 doi:10.1073/pnas.0801921105.
- 1319 Walke, J. B., Harris, R. N., Reinert, L. K., Rollins-Smith, L. A., and Woodhams, D. C. (2011). Social  
1320 immunity in amphibians: evidence for vertical transmission of innate defenses. *Biotropica* 43, 396–  
1321 400. doi:10.1111/j.1744-7429.2011.00787.x.
- 1322 Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014).  
1323 Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 1–11.  
1324 doi:10.1038/ismej.2014.77.
- 1325 Walke, J. B., Becker, M. H., Hughey, M. C., Swartwout, M. C., Jensen, R. V., and Belden, L. K.  
1326 (2015). Most of the dominant members of amphibian skin bacterial communities can be readily  
1327 cultured. *Appl. Environ. Microbiol.*, doi:10.1128/AEM.01486-15.
- 1328 Watve, M., Shejval, V., Sonawane, C., Rahalkar, M., Matapurkar, A., Shouche, Y., et al. (2000). The  
1329 “K” selected oligotrophic bacteria: A key to uncultured diversity? *Curr. Sci.* 78, 1535–1542.
- 1330 Weiss, S. J., Xu, Z., Amir, A., Peddada, S., Bittinger, K., Gonzalez, A., et al. (2015). Effects of  
1331 library size variance, sparsity, and compositionality on the analysis of microbiome data. *PeerJ*.  
1332 3:e1408 <https://dx.doi.org/10.7287/peerj.preprints.1157v1>  
1333
- 1334 Wiggins, P.J., Smith, J.M., Harris, R.N., Minbiole, K.P.C. (2011). The gut of red-backed salamanders  
1335 (*Plethodon cinereus*) may serve as a reservoir for an antifungal cutaneous bacterium. *Journal of*  
1336 *Herpetology*, 45, 329-332.
- 1337 Wishart, D. S., Tzur, D., Knox, C., Eisner, R., Guo, A. C., Young, N., et al. (2007). HMDB: The  
1338 human metabolome database. *Nucleic Acids Res.* 35, 521–526. doi:10.1093/nar/gkl923.
- 1339 Wishart, D. S., Knox, C., Guo, A. C., Eisner, R., Young, N., Gautam, B., et al. (2009). HMDB: A  
1340 knowledgebase for the human metabolome. *Nucleic Acids Res.* 37, 603–610.  
1341 doi:10.1093/nar/gkn810.
- 1342 Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M., Knox, C., Liu, Y., et al. (2013). HMDB 3.0-The  
1343 Human Metabolome Database in 2013. *Nucleic Acids Res.* 41, 801–807. doi:10.1093/nar/gks1065.
- 1344 Woodhams, D. C., Vredenburg, V. T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., Briggs,  
1345 C. J., et al. (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened  
1346 mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398.  
1347 doi:10.1016/j.biocon.2007.05.004.
- 1348 Woodhams, D., Alford, R. A., and Briggs, C. J. (2008). Life-history trade-offs influence disease in  
1349 changing climates: strategies of an amphibian pathogen. *Ecology.* 89, 1627–1639.  
1350 <http://www.esajournals.org/doi/abs/10.1890/06-1842.1>.

### Using multi-omics for probiotic selection

- 1351 Woodhams, D. C., Bigler, L., and Marschang, R. (2012). Tolerance of fungal infection in European  
1352 water frogs exposed to *Batrachochytrium dendrobatidis* after experimental reduction of innate  
1353 immune defenses. *BMC Vet. Res.* 8, 197. doi:10.1186/1746-6148-8-197.
- 1354 Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., et al. (2014).  
1355 Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and  
1356 probiotic effectiveness. *PLoS One* 9, e96375. doi:10.1371/journal.pone.0096375.
- 1357 Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al.  
1358 (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against  
1359 emerging fungal pathogens. *Ecology*. 96, 595–595. doi:10.1890/14-1837.1.
- 1360 Woyke, T., Teeling, H., Ivanova, N. N., Huntemann, M., Richter, M., Gloeckner, F. O., et al. (2006).  
1361 Symbiosis insights through metagenomic analysis of a microbial consortium. *Nature*. 443, 950–955.  
1362 doi:10.1038/nature05192.
- 1363 Xia, L. C., Ai, D., Cram, J., Fuhrman, J. A., and Sun, F. (2013). Efficient statistical significance  
1364 approximation for local similarity analysis of high-throughput time series data. *Bioinformatics*. 29,  
1365 230–237. doi:10.1093/bioinformatics/bts668.
- 1366 Xu, Z., Hansen, M. A., Hansen, L. H., Jacquioid, S., and Sørensen, S. J. (2014). Bioinformatic  
1367 approaches reveal metagenomic characterization of soil microbial community. *PLoS One* 9, e93445.  
1368 doi:10.1371/journal.pone.0093445.
- 1369 Yap, B. T. A., Koo, M. S., Ambrose, R. F., Wake, D. B., and Vredenburg, V. T. (2015). Averting a  
1370 North American biodiversity crisis. *Science*. 349, 481–482.
- 1371 Zeng, B., Han, S., Wang, P., Wen, B., Jian, W., Guo, W., et al. (2015). The bacterial communities  
1372 associated with fecal types and body weight of rex rabbits. *Sci. Rep.* 5, 9342. doi:10.1038/srep09342.
- 1373 Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E. J., Short, J. M., et al. (2002). Cultivating  
1374 the uncultured. *Proc. Natl. Acad. Sci. U. S. A.* 99, 15681–15686. doi:10.1073/pnas.252630999.
- 1375 Zhou, J., Deng, Y., Luo, F., He, Z., and Yang, Y. (2011). Phylogenetic molecular ecological network  
1376 of soil microbial communities in response to elevated CO<sub>2</sub>. *MBio* 2:4, e00122–11.  
1377 doi:10.1128/mBio.00122-11.