# Diversity Predicts Ability of Bacterial Consortia to Mitigate a Lethal Wildlife Pathogen

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**Running Title:**

Bacterial diversity alters pathogen growth

**Conflict of Interest:**

The authors declare no conflict of interest.

**ABSTRACT**

Symbiotic bacterial communities can protect their hosts from infection by pathogens. Treatment of wild individuals with protective bacteria isolated from hosts can combat the spread of emerging infectious diseases, but it is unclear whether the degree of bacterially-mediated host protection is uniform across multiple isolates of globally-distributed pathogens. Here we use the lethal amphibian fungal pathogen *Batrachochytrium dendrobatidis* as a model to investigate the traits predicting broad-scale *in vitro* inhibitory capabilities of both individual bacteria and multiple-bacterial consortia. We show that inhibition of multiple pathogen isolates is rare, with no clear phylogenetic signal at the genus level. Bacterial consortia offer stronger protection against *B. dendrobatidis* compared to single isolates, but critically this was only true for consortia containing multiple genera, and this pattern was not uniform across all *B. dendrobatidis* isolates. These novel insights have important implications for the effective design of bacterial probiotics to mitigate emerging infectious diseases.

## INTRODUCTION

The last 50 years have seen the emergence of several virulent wildlife pathogens with broad host ranges (Tompkins et al 2015). These emerging infectious disease (EIDs) have decimated wildlife populations globally, and are a major contributor to the current global loss of biodiversity (e.g. Skerratt et al 2007; McCallum 2012). Both climate change (Cohen et al 2017) and the global trade in animals (Tompkins et al 2015) are exacerbating the spread of EIDs, and broad-scale, effective treatments and/or prophylaxis for these pathogens in the wild are often lacking (Sleeman 2013; Garner et al 2016). Developing such treatments is often complicated by broad variation in genetic and phenotypic traits such as virulence exhibited by these pathogens (e.g. de Jong & Hien 2006; Schock et al 2010; Farrer et al 2011). Successful mitigation of EIDs in the wild demands that preventative or curative therapies demonstrate broad activity over as many genetic variants of the pathogen as possible, and developing mitigation strategies that satisfy this criterion remains a major outstanding research goal.

Most EIDs are attributed to fungal pathogens, including *Pseudogymnoascus destructans* that causes white nose syndrome in bats, and *Batrachochytrium spp*., which causes chytridiomycosis in amphibians (Fisher et al 2012). *Batrachochytrium dendrobatidis* comprises multiple, deeply diverged lineages, and is capable of rapid evolution (Farrer et al 2011; 2013). Endemic hypovirulent lineages of *B. dendrobatidis* have been identified, including *Bd*CAPE (South Africa), *Bd*CH (Switzerland), *Bd*Brazil (Brazil) and a lineage from Japan (Goka et al 2009; Farrer et al 2011; Schloegel et al 2012; Rosenblum et al 2013; Rodriguez et al 2014), although there are cases where these have spread to other regions and are implicated in population declines in those regions (e.g. *Bd*CAPE in Mallorcan midwife toads, *Alytes muletensis;* Doddington et al 2013). The globally distributed and hypervirulent global panzootic lineage (*Bd*GPL) is the genetic lineage of *B. dendrobatidis* associated with phenomenal mass mortalities and rapid population declines of amphibians around the world, and is a major driver of the current “amphibian extinction crisis” (Fisher et al 2009; Farrer et al 2011; Olson et al 2012). Isolates within this lineage exhibit enormous and unpredictable variation in virulence, even within a single host species exposed under laboratory conditions (Farrer et al 2011; Farrer et al 2013). There is currently no cure for this disease in the wild (reviewed in Garner et al 2016), and given that amphibian communities may be host to multiple *Bd*GPL genotypes (Morgan et al 2007; Rodriguez et al 2014), and that continuous global movement of humans and wildlife transports the fungus, any

67 prophylactic or curative treatment needs to effective against multiple *B. dendrobatidis* genotypes and
68 isolates.

69 Bacterial probiotics represent a promising tool to combat major emerging fungal pathogens in the wild,
70 including *Pseudogymnoascus destructans* (Hoyt et al 2015), *B. dendrobatidis*, and the closely related
71 *B. salamandrivorans* (Martel et al 2013; 2014). Of these, probiotic research is currently most advanced
72 for *B. dendrobatidis* (reviewed in Bletz et al 2013 and Rebollar et al 2016). Laboratory and field studies
73 have shown host-associated bacterial communities (hereafter referred to as the 'microbiome') protect
74 amphibians from *B. dendrobatidis* infection, and that it is possible to artificially augment the
75 microbiome with 'probiotic' bacteria to improve survivorship in response to the pathogen (Bletz et al
76 2013; Becker et al 2015; Walke et al 2015).

77 To date, most *in vitro Bd*GPL challenge experiments have tested the ability of candidate probiotics to
78 limit the growth just a single isolate of *Bd*GPL. This is problematic because the inhibitory capabilities of
79 individual bacteria are not uniform across the variation presented by *Bd*GPL (Antwis et al 2015).
80 Previous work has found no evidence of a phylogenetic signal in the ability of bacterial genera to
81 inhibit a singular *Bd*GPL isolate (Becker et al 2015), but a major gap in our understanding concerns
82 whether some bacterial genera are more likely to show broad-spectrum inhibition across a range of
83 *Bd*GPLs, allowing a more focussed search for effective amphibian probiotics. Furthermore, both *in*
84 *vivo* amphibian probiotic trials and i*n vitro* challenges focus on the application of a singular bacterial
85 isolate to arrest the growth of *B. dendrobatidis*, yet the importance of a complex and diverse
86 microbiome for resilience to infection has been repeatedly demonstrated across a range of host taxa
87 (e.g. Dillon et al 2005; Matos et al 2005; Van Elsas et al 2012; Eisenhauer at el 2013). A novel
88 alternative strategy involves a 'bacterial consortium' approach to probiotics, whereby multiple inhibitory
89 bacterial isolates are applied simultaneously. Multi-species consortia can increase the inhibition of
90 *Bd*GPL (Piova-Scott et al 2017), and so may offer greater inhibitory capabilities across a wider range
91 of *B. dendrobatidis* isolates, however the generality of this pattern across multiple pathogen variants
92 remains untested. Addressing the shortfall in our understanding is critical for developing effective tools
93 for the mitigation of EIDs in the wild.

94 Here we extend previous work to quantify the ability of metabolites from both individual bacteria and
95 co-cultured bacterial consortia to demonstrate broad-scale inhibition across a panel of *B.*
96 *dendrobatidis* isolates. First, we test 58 bacterial isolates from 10 genera for inhibition against a suite

of 10 different *Bd*GPL isolates to quantify; i) variation among bacterial genera in ability to demonstrate broad-spectrum *Bd*GPL inhibition; and ii) variation among *Bd*GPL isolates in susceptibility to inhibition. Second, we quantify the relative efficacy of using single bacterial isolates or bacterial consortia to modify *B. dendrobatidis* growth rates *in vitro*. Specifically, we investigate; iii) whether consortia yield stronger inhibition than single bacteria across three *B. dendrobatidis* isolates from two lineages (*Bd*GPL and *Bd*CAPE); and iv) whether the diversity of a bacterial consortium (number of member genera) affects inhibitory capabilities.

## METHODS

### *Phylogeny screening*

*In vitro* challenges were conducted for 58 bacteria isolated from wild *Agalychnis spp.* frogs in Belize (Antwis et al 2015) to screen for inhibitory capabilities against 10 *Bd*GPL isolates (Table 1, Figure 1). Bacteria belonged to 10 genera, with 3-11 bacterial isolates per genus (Table S1). Bacteria were previously identified (Antwis et al 2015) using colony PCR with primer pair 27F and 1492R for the 16S rRNA gene, which were sequenced at the University of Manchester, and then the forward and reverse sequences were aligned for each bacterium and blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Inhibition challenges were conducted using an *in vitro* spectrophotometer assay method adapted from Bell et al. (2013), Woodhams et al (2014) and Becker et al (2015). Bacteria were grown by adding 50ul of frozen stock bacteria (stored in 30% glycerol, 70% tryptone solution at -80°C) to 15ml of 1% tryptone, and incubating at 18°C for 36 hours until turbid (three cultures per bacterial isolate). Although cell density has been shown to influence metabolite production in culture (Yasumiba et al 2015), we decided not to count and adjust cell density prior to inhibition trials as subsequent addition of media may alter the metabolite profiles already produced by cultures. In addition, cultures were not grown in the presence of *B. dendrobatidis* as multiple *B. dendrobatidis* isolates were tested in this study and this would have confounded results.

Turbid cultures were filtered through a 0.22um sterile filter (Millipore, Ireland) to remove live cells, leaving only bacterial metabolites in solution. These were then combined across the three cultures for a given bacterial isolate, and kept on ice until *B. dendrobatidis* challenges were conducted. *Bd*GPL (Table 1) isolates were grown in 1% tryptone broth until maximum zoospore production was observed

126 (~3-4 days; ~1 x $10^6$ zoospores $ml^{-1}$). As with bacteria, three flasks per *B. dendrobatidis* isolate were
127 grown and then combined prior to challenges to limit flask-effect. Zoospores were separated from
128 sporangia by filtering through 20um sterile filters (Millipore, Ireland). To conduct the
129 spectrophotometer assays, 50ul of bacterial metabolites and 50ul of *B. dendrobatidis* suspension were
130 pipetted into 96-well plates. Each *B. dendrobatidis*-bacteria combination was run with three repeats.
131 Positive controls were included using 50ul 1% tryptone instead of bacterial metabolites. Negative
132 controls were included using 50ul sterile water and 50ul of heat-treated *B. dendrobatidis* for each
133 isolate. Plate readings were taken every 24 hours for four days using a 492nm filter.

134 For each measurement, data were transformed using the equation Ln(OD/(1-OD)), and a regression
135 analysis was used to gain the slope values for each sample over time. Slopes of triplicate replicates
136 for each Bd/Bacteria combination were averaged, and total *B. dendrobatidis* inhibition was calculated
137 using the formula: Inhibition (%) = [1-(slope of sample/slope of control)] x 100. A positive value
138 represents inhibition of *B. dendrobatidis* growth, and a negative value indicates enhanced growth of *B.*
139 *dendrobatidis*.

140

141 *Bacterial consortium challenges*

142 Three bacteria were then selected from each of four genera (*Acinetobacteria, Chryseobacterium,*
143 *Serratia, Stenotrophomonas*) based on their inhibition profiles; poor to medium inhibitors were
144 selected to determine whether combining these bacteria would improve their inhibitory capabilities.
145 Bacteria were grown individually until turbid and added to fresh tryptone either individually (strains A,
146 B and C of each genus separately), or as a triple (strains A, B and C of each genus together to form
147 single-genus mixes, or a random combination of strains across genera to form multi-genus consortia
148 (Table 2)). For both individual and triple bacterial combinations, a total of 3ml of bacteria were added
149 to 12ml of fresh 1% tryptone broth and left to grow together for 12 hours. The volume of each
150 bacterium added depended on whether the consortium contained one or three bacteria, and the
151 volume was split evenly between the number of bacteria added to each group. Following this,
152 bacteria-*B. dendrobatidis* challenges were conducted using the same methods as described above
153 against three *B. dendrobatidis* isolates (Table 1). Average inhibition percentages for each consortium-
154 *B. dendrobatidis* combination were calculated as described above.

155

156 *Statistical Analysis*

157 All statistical analyses were conducted in the software R v.3.3.2 (R Core Team 2016; Supplementary
158 Information).

159

160 *Phylogeny Data:* To quantify differences among genera in proportion of *Bd*GPL isolates inhibited
161 (inhibition score >0), we fitted a Binomial GLM with the proportion of the 10 *Bd*GPL isolates each
162 bacterial isolate inhibited as the response, and genus as a fixed effect. We used the quasibinomial
163 error structure as the model was overdispersed (dispersion 6.4), and tested the model containing a
164 genus term with the reduced intercept-only model using a likelihood ratio test.

165 To quantify differences among genera in the *degree* of inhibition (size of inhibition score), we fitted a
166 hierarchical model in the R package *MCMCglmm* (Hadfield 2010) with the individual inhibition scores
167 of each bacterial isolate (n=58) for each *Bd*GPL isolate (n=10; total n = 580) as a Gaussian response.
168 We fitted both *Bd*GPL isolate, and bacterial strain ID nested within bacterial genus as random effects.
169 We use uninformative, parameter-expanded priors for the random effects as detailed in Hadfield
170 (2010). We ran models for a total of 100,000 iterations following a burn-in of 10,000 iterations and
171 using a thinning interval of 50. Posterior model checks indicated no significant autocorrelation within
172 chains (all values < 0.05) and adequate convergence using the Geweke diagnostic (Geweke 1992).
173 Inspection of model residuals from the frequentist analogue of this model fitted in *lme4* (Bates et al
174 2015) revealed normally-distributed residuals and no evidence of heteroscedasticity. Rerunning
175 models with stronger priors has no effect on model results.

176 To calculate % variance in inhibition explained by *Bd*GPL isolate, bacterial genus, and bacterial strain
177 respectively, we extracted the variance components from the variance-covariance matrix of the model
178 above. We expressed the variance of a component *V* as a percentage of the total variance calculated
179 as ($V_{BdGPL} + V_{genus} + V_{strain} + V_{residual}$). We calculated both mean and 95% credible intervals using the
180 posterior samples from the model. To construct Figs. 1 and 2, we extracted the marginal means and
181 95% credible intervals for each bacterial strain and *Bd*GPL isolate, respectively. That is, the bacterial
182 strain modes are marginalised with respect to *Bd*GPL and vice versa, to quantify whether the *average*
183 scores for each *Bd*GPL or bacterial isolate are significantly different from zero.

184 *Consortium Data:* To calculate the relative mean inhibition of single-genus (SG) vs multi-genus (MG)
185 consortia, we fitted a mixed model in *MCMCglmm* with inhibition as a Gaussian response, consortium
186 type as a 2-level factor, and a random effect of *B. dendrobatidis* and using uninformative priors. To
187 calculate whether consortia exhibited stronger inhibition than the mean of their individual isolates, we
188 constructed a binary variable with outcome 1 if a consortium's inhibition was greater than the single
189 isolate mean and 0 if equal to or lower. We fitted this as a response in a binary GLMM with consortium
190 type as a fixed effect, *B. dendrobatidis* as a random effect and using uninformative priors. Neither
191 model exhibited signs of autocorrelation and Geweke statistics for both models indicated
192 convergence.

193 *In silico Probiotic Consortium Trials*: To probe the relative effectiveness of single bacteria, SG
194 consortia and MG consortia (hereafter 'probiotic types') for modifying the growth rates of *B.*
195 *dendrobatidis*, we ran three sets of simulations, each comprising 1000 iterations. For each set of
196 simulations, we calculated i) the proportion of times a MG consortium yielded higher inhibition than a
197 SG consortium; ii) the proportion of times a MG consortium yielded higher inhibition than a single
198 bacterial isolate; iii) the probability that a MG, SG or single bacterial isolate would yield at least 50%
199 inhibition, which we class as strong inhibition. Adopting a Monte Carlo Integration approach allows us
200 to investigate the performance of different probiotic strategies for individual *Bd* isolates. Calculating
201 group means of each probiotic type does not allow one to calculate the frequency that one probiotic
202 type might outperform another, as this approach does not explicitly make pairwise comparisons and in
203 fact loses information by comparing group means. Group means can be skewed by large individual
204 values, and therefore be misleading with respect to the efficacy of a particular strategy if the mean of
205 that group is not reflective of the true variance in the data. However we report group means alongside
206 these statistics where appropriate for comparison. We derived 95% confidence intervals for each test
207 statistic by performing 10,000 bootstrap samples with replacement from the test distributions. The
208 three simulations were as follows:

209 (1) Averaged over all *B. dendrobatidis* isolates: For each iteration, we randomly selected a *B.*
210 *dendrobatidis* isolate, and then randomly selected both a SG and a MG consortium. A Single bacterial
211 isolate score was then selected randomly from one of the members of the MG consortium.

212 (2) *B. dendrobatidis* specific scores: To investigate the potential for the effectiveness of consortia to
213 differ depending on *B. dendrobatidis* isolate, we repeated the simulations as in (1) but performed 1000
214 simulations for *each B. dendrobatidis* isolate.

215 (3) Sequential *B. dendrobatidis* exposure: Finally, we examined the ability of the three probiotic types
216 to inhibit two *B. dendrobatidis* isolates encountered in series by randomly selecting two of the three *B.*
217 *dendrobatidis* isolates. We assumed that the two isolates are not encountered simultaneously, as co-
218 occurrence of two *Bd* isolates may modify their growth rates and/or a bacterial isolate's ability to inhibit
219 them. For each iteration, we selected a random MG and SG consortium, followed by a randomly-
220 selected single isolate member from the MG consortium. Individual inhibition scores for these three
221 groups were then extracted for both selected *B.* dendrobatidis isolates (i.e. probiotic ID was kept
222 consistent over both pathogen isolates). We calculated the probability that the MG consortium would
223 yield superior inhibition to the SG consortium and single bacterial isolate across both *B. dendrobatidis*
224 isolates, and the probability that all three probiotic types would yield >50% inhibition.

225
226

227 **RESULTS**

228 **Phylogenetic Signals of *Bd*GPL Inhibition**

229 We assayed the ability of 58 bacterial isolates from 10 genera to modify the growth rates of 10 *Bd*GPL
230 isolates. Mean inhibition scores ranged from100% (complete inhibition of growth) to -225% (strong
231 facilitation of growth). At the genus level, there was no significant variation among genera in mean
232 proportion of *Bd*GPL isolates inhibited (Binomial GLM; $\chi^2_9$ = 6.2, p=0.72; Table 3). Six isolates from
233 five genera showed at least weak inhibition across all 10 *Bd*GPLs, whilst seven isolates from five
234 genera facilitated the growth of all 10 isolates (Supplementary Table S1).

235 Variance component analysis revealed considerably more variation in inhibition scores among
236 bacterial strains *within* genera than among genera themselves (Fig. 1). Variation among bacterial
237 strains within genera explained 51% [95% credible interval (CRI) 37-63%] of the variation in *Bd*GPL
238 inhibition scores compared to just 1.3% [0.09-6%] for bacterial genus. *Bd*GPL isolate explained 15.6%
239 [4.8-30%] of the variation in inhibition scores and highlighted two isolates whose marginal effect sizes
240 were significantly negative (JEL423 and AUL2), and one isolate with a significantly positive
241 marginalised inhibition score (08MG04; Fig. 2). JEL423 and AUL2 therefore exhibit strongly enhanced

growth in the presence of bacterial metabolites, whereas 08MG04 is particularly susceptible to inhibition of growth. The remaining seven *Bd*GPL isolates demonstrated no evidence of systematic susceptibility to inhibition of their growth rates across the bacteria tested (Fig. 2).

**Multi-Isolate Consortia as Tools for Pathogen Mitigation**

Consortia containing isolates from Multi-Genus (MG) exhibited significantly higher mean inhibition scores compared to Single-Genus (SG) consortia when marginalising with respect to *B. dendrobatidis* isolate (MG consortia mean inhibition: 36.88%; SG consortia mean: 16.9%; 95% CRI of difference 4.12 – 36.52%, $p_{MCMC}$ = 0.02; Fig. 3). If the ability of a consortium to inhibit *B. dendrobatidis* was simply an additive function of the inhibitory capabilities of the individual bacteria it comprised, we would expect the consortium's inhibition score to be equal to the mean of the individual inhibition scores, weighted by relative abundance. Inhibition scores of consortia greater than the mean of individual isolate scores are indicative of synergistic effects, whereby the combined pool of metabolites from multiple bacteria inhibits *B. dendrobatidis* more strongly than the individual isolates. MG consortia had a 61% probability of demonstrating stronger inhibition than the mean of their single composite bacterial isolates, which was significantly higher than the corresponding probability for SG isolates (26.6%, Mean difference 39.4% [95% Credible Interval 11.2-65.1%], $p_{MCMC}$ = 0.01).

***in silico Probiotic Consortia Trials***

Of the 1000 simulated probiotic trials, naïve application of a MG consortium yielded higher *B. dendrobatidis* inhibition in 69.4% of cases [95% CI 66.5-72.3%] compared to SG consortia (null expectation 50%, $p_{RAND}$<0.001). Moreover, MG consortia had a 38.1% [35.1 – 41.1%] probability of yielding inhibition greater than 50% (strong inhibition), compared to only 13.9% [11.8 – 16.1%] probability for SG consortia. Mean inhibition for all MG consortia was 36.7%, compared to 16.47% for SG consortia. MG consortia outperformed the single isolate in 61% [58-64%] of cases (null expectation 50%, $p_{RAND}$<0.001). However, by averaging over all *B. dendrobatidis* isolates, these results masked substantial variation among *B. dendrobatidis* isolates in the relative efficacy of MG versus SG consortia. We repeated the above simulations separately for each *B. dendrobatidis* isolate, and found that MG consortia were superior to SG consortia and single bacterial isolates for only two *B.*

271 *dendrobatidis* isolates (*Bd*GPL MODS28 and *Bd*CAPE TF5a1), and performed slightly worse than SG
272 consortia for *Bd*GPL SFBC019 (Fig. 4A). Moreover, although MG consortia have the greatest
273 probability of yielding >50% inhibition for *Bd*GPL MODS28 and *Bd*CAPE TF5a1, this was not the case
274 for *Bd*GPL SFBC019, where SG consortia had a marginally higher probability of delivering strong
275 inhibition (Fig. 4B).

276 Finally*, we* tested the ability of both MG and SG consortia to inhibit the growth of two different *B.*
277 *dendrobatidis* isolates in series, as individuals in a single location may be exposed to multiple variants
278 of a pathogen (Goka et al 2009; Schloegel et al 2012; Rodriguez et al 2014; Jenkinson et al 2016), or
279 strong spatial structure of the pathogen and high host dispersal may expose individuals to multiple
280 pathogen variants consecutively. For a given trial, the modelling outcomes were; i) MG consortia
281 inhibited both *B. dendrobatidis* isolates more strongly than SG consortia; ii) SG consortia inhibited
282 both *B. dendrobatidis* isolates more strongly than MG consortia; iii) MG inhibited the first *B.*
283 *dendrobatidis* isolate more strongly than SG consortia, but not the second; iv) MG inhibited the second
284 *B. dendrobatidis* isolate more strongly than SG consortia, but not the first. Applying the same MG
285 consortium to two *B. dendrobatidis* isolates in series achieved stronger inhibition than SG consortia in
286 49.4% [46.3 – 52.5%] of cases (i.e. modelling outcome i; null expectation 25% [$0.5^2$], $p_{RAND}$<0.001).
287 This compared to only 7.9% [6.4-9.6%] of cases where SG consortia exhibited superior inhibition for
288 both *B. dendrobatidis* isolates (i.e. modelling outcome iv). Mean inhibition across both *Bd* isolates for
289 the MG consortia was 73.4%, compared to 32.5% for SG consortia. MG consortia provided superior
290 inhibition for only one of the *B. dendrobatidis* isolates in the remaining 43% of cases (mean 20.3% and
291 22.4% of simulations with superior inhibition for the first and second isolate respectively). MG
292 consortia exhibited strong inhibition (>50%) for both isolates in 14.7% [12.5-17%] of cases, compared
293 to zero cases where SG isolates did so. Applying a single bacterial isolate instead of a SG or MG
294 consortium resulted in strong inhibition for both *B. dendrobatidis* isolates in only 4% [2.9-5.3%] of
295 cases (Fig. 4C).

296

297 **DISCUSSION**

298 The principal objectives of this study were two-fold: i) to determine the magnitude, if any, of
299 phylogenetic signal in the ability of certain genera of bacteria to inhibit a broad range of *Bd*GPL

300 isolates; and ii) to examine the relative effectiveness of single bacteria and bacterial consortia to inhibit
301 several isolates of *B. dendrobatidis*. We found no evidence of variation among bacterial genera in their
302 ability to exhibit broad-range inhibition across multiple *Bd*GPL isolates. Furthermore, our data
303 suggested consortia provide superior *B. dendrobatidis* inhibition than individual bacteria, but critically
304 this pattern is not uniform across pathogen isolates, and is contingent on consortium taxonomic
305 diversity. Our results have important implications for our understanding of the factors determining *in*
306 *vivo* resistance to infection in the wild, and provide novel insights into effective strategies for designing
307 probiotic therapies to mitigate lethal cutaneous infections.

308

309 *Phylogenetic Signals of* Bd*GPL Inhibition*

310 We detected no phylogenetic signal in the ability of individual bacterial genera to inhibit multiple
311 *Bd*GPL isolates. These data support previous work suggesting the ability to inhibit *B. dendrobatidis* is
312 distributed widely over bacterial genera (Antwis et al 2015; Becker et al 2015); several isolates
313 demonstrated at least weak inhibition for all 10 *Bd*GPLs but were spread across multiple genera with
314 no clear pattern. That there is clear functional redundancy among genera in this host-protective trait
315 suggests it is not prudent to focus on any one genus in the search for beneficial probiotics (Becker et
316 al 2015), as highly divergent microbial communities can still possess similar functional traits (e.g. Bletz
317 et al 2016). The principal source of variance in inhibition was among bacterial strains, with the number
318 of isolates demonstrating broad-spectrum *facilitation* of *Bd*GPL being roughly equal to the number
319 exhibiting broad-scale *inhibition* of the pathogen. The phenomenon of *Bd*GPL growth facilitation has
320 been described previously for single pathogen isolates (Bell et al 2013; Becker et al 2015), but
321 crucially our results suggest that a bacterial strain's ability to facilitate the growth of *B. dendrobatidis*
322 may extend across a broad suite of pathogen isolates.

323 It is unclear why some bacterial isolates facilitate *B. dendrobatidis* growth, but one likely explanation is
324 that certain bacterial metabolites can act as growth substrates for fungi (Garbaye 1994; Hardoim et al
325 2015), or that different bacterial metabolites alter the abiotic environment (e.g. pH) to confer different
326 growth rates (Romanowski et al 2011). Here we have provided some of the first evidence that
327 facilitation of *B. dendrobatidis* growth is not simply a rare phenomenon arising from specific
328 *Bd*GPL/bacterial combinations, but that this is widespread across bacterial isolates, and different

329 *Bd*GPL isolates differ systematically in their growth rates when exposed to bacterial metabolites. That
330 said, all four CORN isolates showed similar levels of inhibition across all bacterial isolates, whereas
331 the two AUL isolates exhibited markedly different inhibition profiles (Figure 2). We identified one
332 *Bd*GPL isolate that was significantly prone to inhibition, and a further two isolates that demonstrated
333 strong resistance to inhibition across the 58 bacterial isolates we tested. That there is variation in this
334 trait among *Bd*GPL isolates is intriguing; if facilitation occurs because *B. dendrobatidis* uses bacterial
335 metabolites for nutrition, it may suggest some *B. dendrobatidis* variants can use those metabolites
336 more efficiently for growth. Data gathered from additional isolates will allow us to formally test this
337 hypothesis by probing whether a *Bd*GPL's susceptibility to inhibition or facilitation correlates with
338 virulence. Previous work has shown no among-isolate variation in susceptibility of *B. dendrobatidis* to
339 an echinocandin antifungal drug (Fisher et al 2009), yet our data suggest this pattern is not the same
340 for bacterial metabolites. Recombination among lineages of *Bd*GPL is common (Farrer et al 2011),
341 providing a mechanism whereby metabolic genes favouring enhanced growth may be spread following
342 contact among lineages. Our data have two important implications given the proclivity of *B.*
343 *dendrobatidis* for recombination. First, among-isolate variation in susceptibility to inhibition suggests
344 that the relative efficacy of probiotic or curative therapies in the wild will be modified by local *B.*
345 *dendrobatidis* genotype. Second, though we tend to treat bacterial inhibition scores as fixed traits, this
346 ignores the ability of genetic recombination among *B. dendrobatidis* lineages to modify the relationship
347 between bacterial metabolites and pathogen growth rates. Even the application of probiotics
348 themselves may represent a strong selective pressure favouring genetic variants of *B. dendrobatidis*
349 that lack susceptibility to those probiotics. Although several trials have demonstrated the potential for
350 probiotic prophylaxis against *B. dendrobatidis* (e.g. Harris 2009; Muletz et al 2012; Loudon et al. 2014;
351 Kueneman et al 2016), we still lack the requisite data to measure selection caused by those trials on
352 the pathogen. *In vitro* experimental evolution assays between pathogen and bacteria may prove the
353 most powerful means for detecting such patterns.

354

355 *Consortium-Based Approaches to Combatting Fungal Pathogens*

356 Our results revealed a positive link between the taxonomic richness of a probiotic consortium and its
357 ability to inhibit *B. dendrobatidis* growth, but crucially this relationship was highly dependent on *B.*
358 *dendrobatidis* isolate. Multi-genus consortia outperformed both single-genus consortia and single

359 bacterial isolates in *B. dendrobatidis* inhibition, and were far more likely to produce strong inhibition of
360 50% or greater, but only for two of the three pathogen variants.

361 The general relationship between inhibition and consortium diversity was in the expected direction; low
362 community relatedness (i.e. high community dissimilarity) and high species richness both increase the
363 resistance of a bacterial community to pathogenic 'invaders' (e.g. Jousset et al 2011; Eisenhauer et al
364 2012, 2013). Furthermore, previous work has linked higher species diversity of probiotic consortia to
365 increased *B. dendrobatidis* inhibition using a single pathogen isolate (Loudon et al 2014; Piova-Scott
366 et al 2017). Superior inhibition from consortia, rather than single isolates, may arise as a by-product of
367 the interference competition over resources created by co-culture (Scheuring & Yu 2012). Thus, even
368 bacteria that are weak inhibitors when grown individually can increase the overall inhibitory power of a
369 consortium by creating a competitive environment that favours greater production of anti-fungal
370 compounds. Functional dissimilarity has been proposed as more important than taxonomic diversity in
371 predicting a community's resilience to invasion (Eisenhauer et al 2013), but may explain why single-
372 genus consortia did not perform as well as multi-genus consortia. In selecting for genetic diversity, we
373 may have been simultaneously selecting for functional diversity not present when co-culturing three
374 members of the same genus.

375 That *B. dendrobatidis* isolate can alter the strength of the relationship between consortium diversity
376 and inhibition is a highly novel finding. Our simulated probiotic trials revealed that for two *B.*
377 *dendrobatidis* isolates, combining bacteria into multi-genus consortia yielded significantly better
378 inhibition than applying one of the member bacteria in isolation. These results provide further support
379 for a synergistic effect of co-culture on inhibition. If multi-genus consortia were no better at inhibition
380 than the mean of their composite members, Monte Carlo integration over all single isolate scores
381 would not have recovered a significant difference between the two groups. Yet, for *Bd*GPL MODS28
382 and *Bd*CAPE TF5a1, multi-genus consortia yielded by far the highest probability of observing strong
383 inhibition of 50% or more. That this pattern was not conserved for *Bd*GPL SFBC019 is perhaps the
384 most intriguing finding. As for *Bd*GPL variants JEL423 and AUL2 in the phylogenetic trials, SFBC019
385 was largely resistant to inhibition, with individual bacterial inhibition scores that were often negative.
386 One possible explanation for the lack of efficacy of consortia against SFBC019 is that the when a
387 variant of *B. dendrobatidis* is resistant to inhibition and/or there is little variation in inhibition, co-culture
388 fails to produce any synergistic inhibitory effects. That is, if a pathogen is highly resistant to most

bacterial metabolites in the first instance, increases in the relative concentrations of those metabolites through co-culture-mediated competition are unlikely to elicit any significant increases in inhibitory capability. The most important consequence of this pattern is that for some pathogenic variants, multi-genus consortia are unlikely to be able to yield high inhibition in cases where individual isolates have failed to do so. Despite the observed variance in success of consortia across *B. dendrobatidis* isolates, our simulation trials revealed that multi-genus consortia offer the best broad-spectrum protection across multiple *B. dendrobatidis* isolates encountered in series. This finding is important; human-mediated spread of *B. dendrobatidis* through the amphibian trade (Fisher & Garner 2007) means we cannot assumise that local populations will be exposed to only one pathogenic variant. Successful mitigation of the pathogen in the wild demands that we employ strategies with the highest broad-spectrum success over multiple pathogen genotypes. Combining bacteria that show high levels of inhibition across multiple *B. dendrobatidis* isolates may further increase the effectiveness of consortia (intermediate inhibitors were selected for this study to allow greater insight into community dynamics).

*Conclusion*

This study adds to a growing body of evidence suggesting that diverse, multi-species consortia may represent powerful disease mitigation tools, offering superior probiotic protection against disease compared to single bacterial isolates. Our work has highlighted that different isolates of a pathogen can modify the strength of inhibition caused by the probiotic, meaning we cannot expect probiotic effectiveness to be uniform across the genetic landscape of the pathogen. Despite the relative merits of multi-genus consortia for mitigating single and multiple *B. dendrobatidis* variants, it remains to be determined how readily these consortia will be able to colonise the host skin *in vivo*. This is crucial for to being able to quantify how applicable inhibition measures derived *in vitro* are to real-world scenarios. Nevertheless, our data highlight the merits of a community-level approach to probiotic mitigation of wildlife disease, which may offer more broad-spectrum host protection in the face of large-scale heterogeneity in pathogen genotype.

**ACKNOWLEDGEMENTS**

This study was partly funded by a North-West University Postdoctoral Research Fellowship awarded to REA. XAH was funded by an Institute of Zoology Research Fellowship. The authors would like to thank Prof Richard Preziosi and Dr Trenton Garner for additional provision of consumables, and Prof Ché Weldon, Dr Trenton Garner and Prof Matthew Fisher for access to *Batrachochytrium dendrobatidis* isolates used in this study.

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**DATA ACCESSIBILITY**

Genbank accession numbers of all bacteria are provided in Supplementary Materials. Data files and R Markdown will be made available on Dryad.

**AUTHOR CONTRIBUTIONS**

RA and XH conceived the study, RA collected the data, XH analysed the data, RA and XH wrote the paper. Both authors contributed equally to this paper.

632 **TABLE LEGENDS**

633 **Table 1**

634 *Batrachochytrium dendrobatidis* isolates used in the study.

635

636 **Table 2**

637 Composition of multi-genus consortia used in the study. Single-genus consortia comprised all three
638 bacterial isolates (A, B and C) for a given genus (*Acinetobacter, Chryseobacterium, Serratia,*
639 *Stentrophomonas*).

640

641 **Table 3**

642 Mean Proportion of 10 *Bd*GPL isolates for which at least weak inhibitory capability was observed,
643 averaged over all bacterial isolates in a genus. 95% CI: 95% confidence intervals from an
644 overdispersion-corrected Binomial GLM.

645

646

647 **FIGURE LEGENDS**

648 **Figure 1. Inhibition scores of 58 bacterial strains from 10 genera when tested against 10**
649 ***Bd*GPL isolates.** Estimates are derived from a Bayesian mixed effects model with bacterial isolate
650 nested within genus, and *Bd*GPL isolate fitted as random effects. Points are conditional modes of the
651 individual isolate random effects, marginalised with respect to *Bd*GPL isolate. Error bars are 95%
652 credible intervals.

653

654 **Figure 2. Inhibition scores of 10 *Bd*GPL isolates.** Estimates are derived from a Bayesian mixed
655 effects model with bacterial isolate nested within genus, and *Bd*GPL isolate fitted as random effects.
656 Points are conditional modes of the individual *Bd*GPL isolate random effects, marginalised with
657 respect to bacterial isolate. Error bars are 95% credible intervals.

658

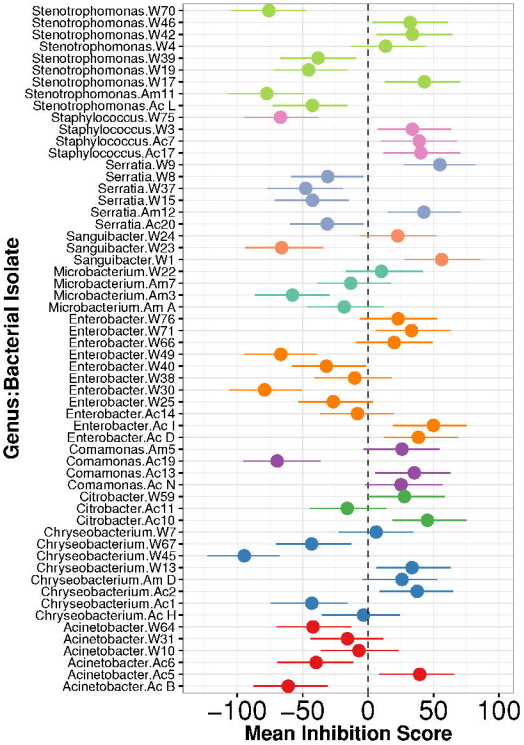
659 **Figure 3. Inhibition scores for Single-Genus and Multi-Genus Consortia across three *B.***
660 ***dendrobatidis* isolates** (*Bd*GPL MODS28.1, *Bd*GPL SFBC019 and *Bd*CAPE TF5a1). Points have
661 been jittered for display purposes.

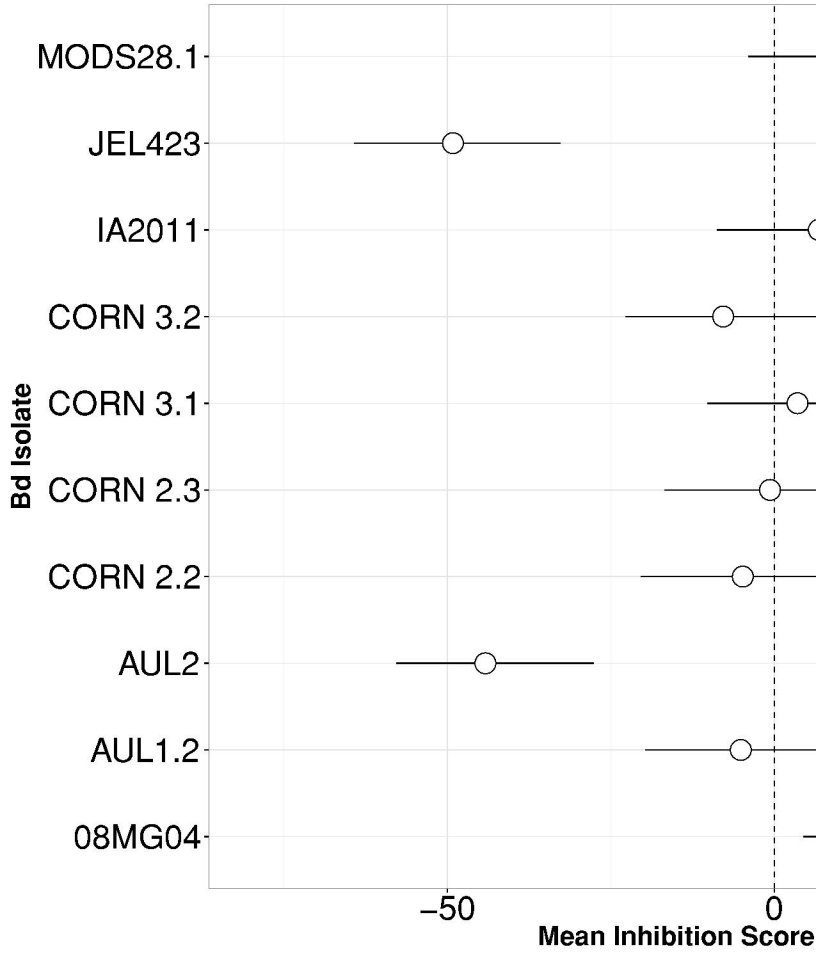
662

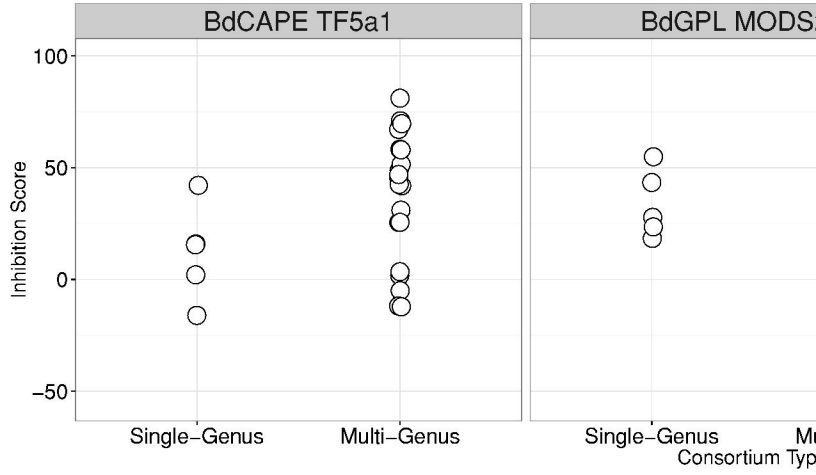
663 **Figure 4. Simulation results examining the relative efficacy of different probiotic strategies.** (A)
664 the probability of Multi-Genus Consortia (MGC) yielding higher inhibition compared to Single-Genus
665 Consortia (SGC) or a Single Bacterial Isolate (Single); (B) the probability of MGC, SGC or Single
666 bacteria yielding inhibition > 50% when applied to each of three *B. dendrobatidis* isolates; (C) The
667 probability of an individual consortium type yielding >50% inhibition when applied to two randomly
668 chosen *B. dendrobatidis* isolates in series.

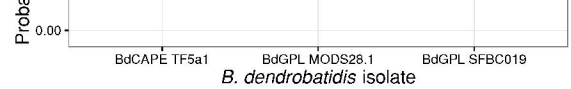
669

670

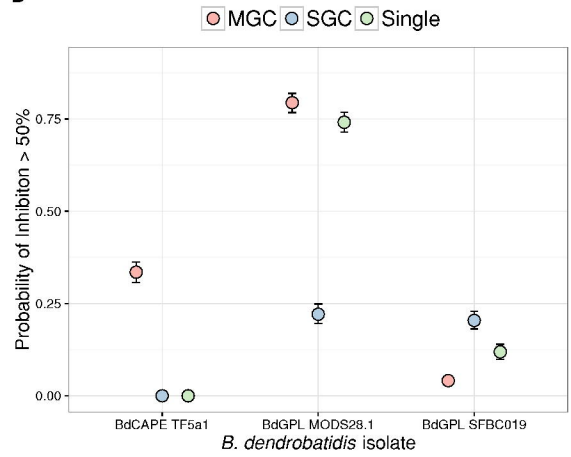

Genus:Bacterial Isolate
Stenotrophomonas.W70
Stenotrophomonas.W46
Stenotrophomonas.W42
Stenotrophomonas.W4
Stenotrophomonas.W39
Stenotrophomonas.W19
Stenotrophomonas.W17
Stenotrophomonas.Am11
Stenotrophomonas.Ac L
Staphylococcus.W75
Staphylococcus.W3
Staphylococcus.Ac7
Staphylococcus.Ac17
Serratia.W9
Serratia.W8
Serratia.W37
Serratia.W15
Serratia.Am12
Serratia.Ac20
Sanguibacter.W24
Sanguibacter.W23
Sanguibacter.W1
Microbacterium.W22
Microbacterium.Am7
Microbacterium.Am3
Microbacterium.Am A
Enterobacter.W76
Enterobacter.W71
Enterobacter.W66
Enterobacter.W49
Enterobacter.W40
Enterobacter.W38
Enterobacter.W30
Enterobacter.W25
Enterobacter.Ac14
Enterobacter.Ac I
Enterobacter.Ac D
Comamonas.Am5
Comamonas.Ac19
Comamonas.Ac13
Comamonas.Ac N
Citrobacter.W59
Citrobacter.Ac11
Citrobacter.Ac10
Chryseobacterium.W7
Chryseobacterium.W67
Chryseobacterium.W45
Chryseobacterium.W13
Chryseobacterium.Am D
Chryseobacterium.Ac2
Chryseobacterium.Ac1
Chryseobacterium.Ac H
Acinetobacter.W64
Acinetobacter.W31
Acinetobacter.W10
Acinetobacter.Ac6
Acinetobacter.Ac5
Acinetobacter.Ac B
−100
−50
0
50
100
Mean Inhibition Score


MODS28.1
JEL423
IA2011
CORN 3.2
CORN 3.1
CORN 2.3
CORN 2.2
AUL2
AUL1.2
08MG04
Bd Isolate
−50
0
Mean Inhibition Score

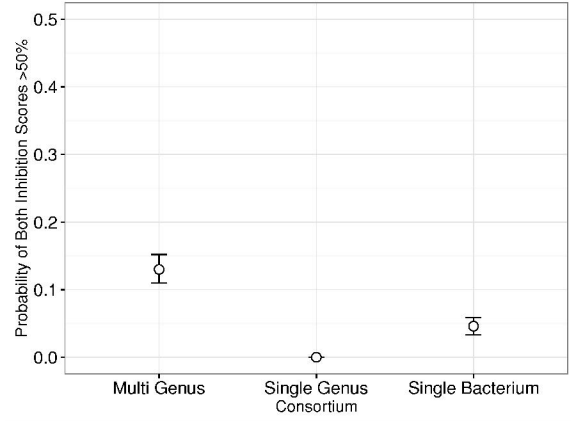

BdCAPE TF5a1
BdGPL MODS
Inhibition Score
100
50
0
−50
Single−Genus
Multi−Genus
Single−Genus
Consortium Typ


Probability
0.00
BdCAPE TF5a1
BdGPL MODS28.1
BdGPL SFBC019
B. dendrobatidis isolate

B


MGC
SGC
Single
Probability of Inhibiton > 50%
0.75
0.50
0.25
0.00
BdCAPE TF5a1
BdGPL MODS28.1
BdGPL SFBC019
B. dendrobatidis isolate

C


Probability of Both Inhibition Scores >50%
0.5
0.4
0.3
0.2
0.1
0.0
Multi Genus
Single Genus Consortium
Single Bacterium