Probiotic Consortia Are Not Uniformly Effective Against Different Amphibian Chytrid Pathogen Isolates

Rachael E. Antwis[1,2* #], Xavier A. Harrison[3 #]

1. School of Environment and Life Sciences, University of Salford, Salford, UK

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 NW1 4RY

 # Both authors contributed equally to this paper

Addresses for Correspondence:

Dr Rachael Antwis, University of Salford, Room 336, Peel Building, The Crescent, Salford, M5 4WT, UK, 01612954641, r.e.antwis@salford.ac.uk

Dr Xavier Harrison, Institute of Zoology, Regents Park, NW1 4RY, UK, xav.harrison@gmail.com

Running Title:

Probiotic diversity alters Bd 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 (probiotics) isolated from hosts can combat the spread of emerging infectious diseases. However, it is unclear whether candidate probiotic bacteria can offer consistent protection across multiple isolates of globally-distributed pathogens. Here we use the lethal amphibian fungal pathogen *Batrachochytrium dendrobatidis* to investigate whether probiotic richness (number of bacteria) or genetic distance among consortia members influences broad-scale *in vitro* inhibitory capabilities of probiotics across multiple isolates of the pathogen. We show that inhibition of multiple pathogen isolates by individual bacteria is rare, with no systematic pattern among bacterial genera in ability to inhibit multiple *B. dendrobatidis* isolates. Bacterial consortia can offer stronger protection against *B. dendrobatidis* compared to single strains, and this tended to be more pronounced for consortia containing multiple genera compared with those consisting of bacteria from a single genus (i.e. with lower genetic distance), but critically this effect 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 diseases have decimated wildlife populations globally and are major contributors to the current global loss of biodiversity (e.g. Skerratt et al 2007; McCallum 2012). Broad-scale treatments and/or prophylaxis for such pathogens are often lacking for wild animals (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 infectious diseases 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.

Batrachochytrium dendrobatidis is a highly infectious fungal pathogen responsible for the global decline in amphibians and a major driver of the current "amphibian extinction crisis" (reviewed in Garner et al 2016). This pathogen comprises multiple deeply diverged lineages and is capable of rapid evolution through extensive genomic recombination (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 these may also be implicated in population declines in novel regions (e.g. *Bd*CAPE in Mallorcan midwife toads, *Alytes muletensis;* Doddington et al 2013). However, it is the globally distributed hypervirulent global panzootic lineage (*Bd*GPL) that is associated with phenomenal mass mortalities and rapid population declines of amphibians around the world (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). Given that amphibian communities may be host to multiple *B. dendrobatidis* variants (Morgan et al 2007; Rodriguez et al 2014) and that global movement of humans and wildlife continues to transport the pathogen (Garner et al 2016), any prophylactic or curative treatment needs to be effective against multiple *B. dendrobatidis* variants.

Bacterial probiotics represent a promising tool to combat emerging infectious diseases in the wild, including *B. dendrobatidis* (Bletz et al 2013, Hoyt et al 2015; Rebollar et al 2016). Laboratory and field studies have shown host-associated bacterial communities protect amphibians from *B. dendrobatidis* infection and that it is possible to artificially augment the microbiota with probiotic bacteria to improve survivorship in response to the pathogen (Harris et al. 2009; Muletz et al. 2012; Bletz et al 2013; Becker et al 2015; Walke et al 2015; Kueneman et al. 2017). However, inhibitory capabilities of individual bacteria are not uniform across the variation presented by *B. dendrobatidis* (Antwis et al 2015; Muletz-Wolz et al 2017; Bletz et al 2017a). In addition, previous work has found either no (Becker et al 2015) or weak evidence (Bletz et al 2017a) of a phylogenetic signal in the ability of bacterial genera to inhibit a singular *B. dendrobatidis* isolate. However, a major gap in our understanding concerns whether some bacterial genera are more likely to show broad-spectrum inhibition across a range of *B. dendrobatidis* isolates, allowing a more focused search for effective amphibian probiotics. Furthermore, the importance of a complex and diverse microbiota for resilience to infection has been repeatedly demonstrated across a range of host taxa (e.g. Dillon et al 2005; Matos et al 2005; Van Elsas et al 2012; Eisenhauer at el 2013). An alternative strategy for probiotic development involves a 'bacterial consortium' approach, whereby multiple inhibitory bacterial strains are applied simultaneously. Multi-species consortia can increase inhibition of *B. dendrobatidis* growth through increased competition and the production of emergent metabolites (Loudon et al 2014; Piova-Scott et al 2017), and may offer greater inhibitory capabilities across a wider range of *B. dendrobatidis* isolates. However, the generality of this pattern across multiple pathogen variants remains untested. Addressing this shortfall in our understanding is critical for developing effective tools for the mitigation of emerging infectious diseases in the wild.

Here we extend previous work to quantify the ability of individual bacteria and co-cultured bacterial consortia to demonstrate broad-scale inhibition across a panel of *B. dendrobatidis* isolates. First, we test 54 bacterial strains 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 strains 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 genus-level diversity of a bacterial consortium affects inhibitory capabilities.

METHODS

Taxonomic Classification

In vitro challenges were conducted for 54 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, Fig. 1). *Batrachochytrium dendrobatidis* is present in the Maya Mountains from where these bacteria were collected, although declines in *Agalychnis* hosts were not seen in this area (Kaiser & Pollinger 2012; Antwis, pers. obvs.). Bacterial strains belonged to 10 genera with 3-11 bacteria per genus (Table S1). Bacteria were identified using colony PCR to amplify the 16S rRNA gene (with primers 27F and 1492R) and sequenced at the University of Manchester (Antwis et al 2015). The forward and reverse sequences were aligned for each bacterium and blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To calculate genetic distance among sequences, we aligned the sequences against the SILVA reference database (Quast et al 2013). We used the *seqinr* package (Charif & Lobry 2007) to import the aligned sequences and calculate the pairwise genetic distances among bacterial strains.

Inhibition challenges were conducted using an *in vitro* absorbance-based growth inhibition assay 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 strain). 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.

Turbid cultures were filtered through a 0.22um sterile filter (Millipore, Ireland) to remove live cells, leaving only bacterial products (including metabolites) in the filtrate. These were then combined across the three cultures for a given bacterial strain 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 (~3-4 days; ~1 x 10^6 zoospores ml^{-1}). As with bacteria, three flasks per *B. dendrobatidis* isolate were grown and then combined prior to challenges to minimise flask-effect. Zoospores were separated from sporangia by filtering through 20um sterile filters (Millipore, Ireland). To conduct the absorbance-based growth inhibition assays, 50ul of bacterial filtrate and 50ul of *B. dendrobatidis* suspension were pipetted into 96-well plates. Each *B. dendrobatidis*-bacteria combination was run in triplicate. Positive controls were included using 50ul 1% tryptone instead of bacterial filtrate. Negative controls were included using 50ul sterile tryptone and 50ul of heat-treated *B. dendrobatidis* for each isolate. Plate readings were taken using a 492nm filter on initial construction of the challenge assays and every 24 hours for four subsequent days.

For each measurement, data were transformed using the equation Ln(OD/(1-OD)) and a regression analysis was used to gain the slope values for each sample over time. Slopes of triplicate replicates for each *B. dendrobatidis*-bacteria combination were averaged, and the slope of the negative controls subtracted. Total *B. dendrobatidis* inhibition was calculated using the formula: Inhibition (%) = [1-(slope of sample/slope of control)] x 100 to give an 'inhibition score'. A positive inhibition score represents inhibition of *B. dendrobatidis* growth and a negative score indicates enhanced growth of *B. dendrobatidis*. It should be noted that we did not use a nutrient depleted control in our experiments (Bell et al 2013), which means *B. dendrobatidis* inhibition relative to the controls may be slightly under-estimated.

Bacterial consortium challenges

Three bacterial strains were then selected from each of five genera (*Chryseobacterium, Comamonas, Enterobacter, Staphlycoccus* and *Stenotrophomonas*) based on their inhibition profiles; poor to medium inhibitors were selected to determine whether combining these bacteria would improve their inhibitory capabilities (mean percentage inhibition score of approximately 0 to +50; Fig. 1). Bacteria were grown individually until turbid and added to fresh tryptone either individually (strains A, B and C of each genus separately), or as a triple (strains A, B and C of each genus together to form five single-genus mixes, or a combination of strains across genera to form multi-genus consortia (20 multi-genus combinations tested)). For both individual and triple bacterial combinations, a total of 3ml of bacteria

were added to 12ml of fresh 1% tryptone broth and left to grow together for 12 hours. The volume of each bacterium added depended on whether the consortium contained one or three bacteria, and the volume was split evenly between the number of bacteria added to each group. Following this, bacteria-*B. dendrobatidis* challenges were conducted using the same methods as described above against three *B. dendrobatidis* isolates (Table 1). Average inhibition percentages for each consortium-*B. dendrobatidis* combination were calculated as described above.

Statistical Analysis

All statistical analyses were conducted in the software R v.3.3.2 (R Core Team 2016).

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

To visualise the distribution of inhibition across bacterial strains and *B. dendrobatidis* variants, we constructed a heatmap using the *pheatmap* package in R (Kolde 2015). To quantify differences among genera in the *degree* of inhibition (size of inhibition score), we fitted a hierarchical model in the R package *MCMCglmm* (Hadfield 2010) with the individual inhibition scores of each bacterial strain (n=54) for each *Bd*GPL isolate (n=10; total n = 540) as a Gaussian response. We fitted both *Bd*GPL isolate and bacterial strain ID nested within bacterial genus as random effects. We also controlled for genetic distance among bacterial strains by passing the bacterial 16S gene tree to the model as a phylogenetic random effect. We use uninformative, parameter-expanded priors for the random effects as detailed in Hadfield (2010). We ran models for a total of 100,000 iterations following a burn-in of 10,000 iterations and using a thinning interval of 50. Inspection of model residuals from the frequentist analogue of this model fitted in *lme4* (Bates et al 2015) revealed normally-distributed residuals and no evidence of heteroscedasticity. Rerunning models with stronger priors has no effect on model results. Gelman-Rubin diagnostic of Markov chains indicated adequate convergence, with all potential scale reduction factors <1.01. We used Bayesian models here, rather than a frequentist analogue, due to

the ease of summarising uncertainty in point estimates of random effect conditional means using 95% credible intervals of Markov chain values. To calculate % variance in inhibition explained by *Bd*GPL isolate, bacterial genus, and bacterial strain respectively, we extracted the variance components from the variance-covariance matrix of the model above. We expressed the variance of a component *V* as a percentage of the total variance calculated as ($V_{BdGPL} + V_{genus} + V_{strain} + V_{residual}$). We calculated both mean and 95% credible intervals using the posterior samples from the model. To construct Figures 1 and 2, we extracted the marginal means and 95% credible intervals for each bacterial strain and *Bd*GPL isolate, respectively. That is, the bacterial strain modes are marginalised with respect to *Bd*GPL, and vice versa, to quantify whether the *average* scores for each *Bd*GPL isolate or bacterial strain are significantly different from zero.

Correlation Between Genetic Distance and Inhibition: For each pair of bacterial strains, we calculated the correlation between the inhibition scores for the ten *B. dendrobatidis* isolates. If more closely related bacterial strains are more likely to have similar inhibition profiles, there should be a negative correlation overall between genetic distance and similarity of inhibition. To test this, we performed a Mantel test using the genetic distance and inhibition score similarity matrices in the R package 'vegan' (Oksanen et al 2015).

Consortium Data: To calculate the relative mean inhibition of single-genus vs multi-genus consortia, we fitted a mixed model in *MCMCglmm* with inhibition as a Gaussian response, consortium type as a 2-level factor, and a random effect of *B. dendrobatidis* isolate using uninformative priors. To calculate whether consortia exhibited stronger inhibition than the mean of their individual strains, we constructed a binary variable with an outcome of 1 if a consortium's inhibition was greater than the single strain mean, and 0 if equal to or lower. We fitted this as a response in a binary GLMM with consortium type as a fixed effect, *B. dendrobatidis* as a random effect and using uninformative priors. Neither model exhibited signs of autocorrelation and Geweke statistics for both models indicated convergence. We calculated mean genetic distance among members of consortia using the genetic distance measures outlined above. We fitted a Bayesian GLM where the percentage inhibition of a consortium was a function of the interaction between the genetic distance among consortia members and the *B. dendrobatidis* isolate identity. Genetic distance was standardised prior to model fitting to remove the correlation between main effects and interactions.

Consortium Randomisations: We used a randomisation approach to probe the relative effectiveness of single bacteria, single-genus consortia and multi-genus consortia (hereafter 'probiotic types') for modifying the growth rates of *B. dendrobatidis*. These randomisations used the 'Taxonomic Group' and 'Consortium' inhibition data from above to explore three different scenarios relevant to the application of probiotics to *B. dendrobatidis*. For each iteration of a randomisation we randomly selected a *B. dendrobatidis* isolate and then extracted the inhibition scores of a randomly chosen single bacterial strain, single-genus consortium, and multi-genus consortium. After 1000 iterations, we calculated i) the proportion of times a multi-genus consortium yielded higher inhibition than a single-genus consortium; ii) the proportion of times a multi-genus consortium yielded higher inhibition than a single bacterial strain; iii) the probability that a multi-genus, single-genus or single bacterial strain would yield at least 50% inhibition, which we classed as strong inhibition. This approach is more powerful than simply calculating differences in group means of each probiotic type, as group means can be skewed by large individual values, and therefore be misleading with respect to the efficacy of a particular strategy if the mean of that group is not reflective of the true variance in the data. However, we report group means alongside these statistics where appropriate for comparison. We derived 95% confidence intervals for each test statistic by performing 10,000 bootstrap samples with replacement from the test distributions. The three scenarios we tested were as follows:

Scenario 1: Averaged over all *B. dendrobatidis* isolates: For each iteration, we randomly selected a *B. dendrobatidis* isolate and then randomly selected both a single-genus and a multi-genus consortium. A single bacterial strain score was then selected randomly from one of the members of the multi-genus consortium.

Scenario 2: *B. dendrobatidis* specific scores: To investigate the potential for the effectiveness of consortia to differ depending on *B. dendrobatidis* isolate, we repeated the randomisation as in Scenario 1 but performed 1000 iterations for *each B. dendrobatidis* isolate.

Scenario 3: Sequential *B. dendrobatidis* exposure: Finally, we examined the ability of the three probiotic types to inhibit two *B. dendrobatidis* isolates encountered in series by randomly selecting two of the three *B. dendrobatidis* isolates. We assumed that the two isolates are not encountered simultaneously as co-occurrence of two *B. dendrobatidis* isolates may modify their growth rates and/or a bacterial strain's ability to inhibit them. For each iteration, we selected a random multi-genus and single-genus consortium, followed by a randomly-selected single strain member from the multi-genus

consortium. Individual inhibition scores for these three groups were then extracted for both selected *B. dendrobatidis* isolates (i.e. probiotic ID was kept consistent over both pathogen isolates). We calculated the probability that the multi-genus consortium would yield superior inhibition to the single-genus consortium and single bacterial strain across both *B. dendrobatidis* isolates, and the probability that all three probiotic types would yield >50% inhibition.

RESULTS

*Bd*GPL Inhibition Within and Among Bacterial Genera

We assayed the ability of 54 bacterial strains from 10 genera to modify the growth rates of 10 *Bd*GPL isolates. Mean inhibition scores ranged from 100 (complete inhibition of growth) to -225 (strong facilitation of growth). There were no significant differences among genera in mean proportion of *Bd*GPL isolates inhibited (Binomial GLMM; $\chi^2_9 = 8.12$, p=0.52; Fig. 1; Table 2). Six strains from six genera showed at least weak inhibition across all 10 *Bd*GPLs, whilst five strains from five genera facilitated the growth of all 10 *B. dendrobatidis* isolates (Supplementary Table S1). We did not find a significant correlation between genetic distance and similarity of inhibition profiles (Mantel test r = -0.027, p = 0.77).

We detected considerably more variation in inhibition scores among bacterial strains within genera than among genera (Fig. 1). Variation among bacterial strains within genera explained 87.9% [95% credible interval (CRI) 80.25-94.47%] of the variation in *Bd*GPL inhibition scores compared to just 0.6% [0.007-3.8%] for bacterial genus. *Bd*GPL isolate explained 3.9% [0.1-8.7%] of the variation in inhibition scores. Heatmap hierarchical clustering of inhibition scores revealed two isolates that demonstrated predominantly enhanced growth in the presence of bacterial filtrates (JEL423 and AUL2; Fig. 2). In some cases, *B. dendrobatidis* isolates from similar locations (e.g. CORN isolates from Cornwall) showed similar clustering of inhibitions scores, whereas others (e.g. AUL isolates from the Pyrenees) showed markedly different inhibition fingerprints (Fig. 2).

Multi-Strain Consortia as Tools for Pathogen Mitigation

Consortia containing strains from multiple genera exhibited significantly higher mean inhibition scores compared to single-genus consortia when marginalising with respect to *B. dendrobatidis* isolate (multi-genus consortia mean inhibition: 36.88%; single-genus consortia mean: 16.9%; 95% CRI of difference 4.12 – 36.52%, p_{MCMC} = 0.02; Fig. 3). Multi-genus consortia had a 61% probability of demonstrating stronger inhibition than the mean of their single composite bacterial strains, which was significantly higher than the corresponding probability for single-genus consortia (26.6%, mean difference 39.4% [95% Credible Interval 11.2-65.1%], p_{MCMC} = 0.01). Mean genetic distance among members of multi-genus consortia was significantly higher than among members of single-genus consortia (multi-genus mean distance = 0.45, single-genus mean =0.11, t = -15.5, p<0.001). Consortia with higher mean genetic distance elicited significantly higher inhibition scores for *B. dendrobatidis* isolates *Bd*CAPE TF5a1 and *Bd*GPL MODS28.1 (pMCMC = 0.009), but not for *Bd*GPL SFBC019, which had a significantly different slope to the other two *B. dendrobatidis* isolates (Fig. 4, pMCMC=0.01).

Probiotic Consortium Randomisations

Scenario 1: Our randomisation tests revealed that multi-genus consortia gave higher inhibition than single-genus consortia in 69.4% of cases when averaging over all *B. dendrobatidis* isolates (null expectation 50%, p_{RAND}<0.001). Multi-genus consortia were more likely to produce inhibition greater than 50% (strong inhibition) (38.1% of iterations) compared to single-genus consortia (13.9% of iterations, p<0.001), and outperformed a randomly chosen single bacterial strain in 61% of cases (null expectation 50%, p_{RAND}<0.001). Mean inhibition for all multi-genus consortia across all *B. dendrobatidis* isolates was 36.7%, compared to 16.47% for single-genus consortia.

Scenario 2: When considering *B. dendrobatidis* isolates individually, multi-genus consortia outperformed single-genus consortia and single bacterial strains for only two of the three isolates (*Bd*GPL MODS28 and *Bd*CAPE TF5a1, but not for *Bd*GPL SFBC019; Fig. 5A). This pattern was also evident when determining the probability of yielding >50% inhibition by consortia (Fig. 5B).

Scenario 3: We also tested the ability of both multi-genus and single-genus consortia to inhibit the growth of two different *B. dendrobatidis* isolates in series, as individuals in a single location may be exposed to multiple variants of a pathogen (Goka et al 2009; Schloegel et al 2012; Rodriguez et al 2014; Jenkinson et al 2016), or strong spatial structure of the pathogen and high host dispersal

may expose individuals to multiple pathogen variants consecutively. Applying the same multi-genus consortium to two different randomly-chosen *B. dendrobatidis* isolates in series achieved stronger inhibition than single-genus consortia in 49.4% of cases (null expectation 25%, $p_{RAND}<0.001$). This compared to only 7.9% of cases where single-genus consortia exhibited superior inhibition for both *B. dendrobatidis* isolates. Multi-genus consortia exhibited strong inhibition (>50%) for both isolates in 14.7% of cases, compared to zero cases where single-genus isolates did so. Applying a single bacterial strain instead of a single-genus or multi-genus consortium resulted in strong inhibition for both *B. dendrobatidis* isolates in only 4% of cases (Fig. 5C). The full results of these randomisations, including confidence intervals for tests, can be found in Supplementary Table S2.

DISCUSSION

The principal objectives of this study were two-fold: i) to determine whether certain genera of bacteria are better able to inhibit a broad range of *Bd*GPL isolates; and ii) to examine the relative effectiveness of single bacteria and bacterial consortia to inhibit multiple isolates of *B. dendrobatidis*. We found no evidence of variation among bacterial genera in their ability to exhibit broad-range inhibition across multiple *Bd*GPL isolates. There was considerable within-genus variation in inhibitory capabilities of bacteria compared to between-genus variation, meaning genus is not a reliable indicator of anti-*B. dendrobatidis* capabilities across multiple isolates of this pathogen. Furthermore, our data suggested consortia can provide superior *B. dendrobatidis* inhibition compared to individual bacteria, and that this is contingent on consortium taxonomic diversity, but critically this pattern is not uniform across pathogen isolates. Our results have important implications for developing effective strategies for designing probiotic therapies to mitigate lethal infectious disease.

BdGPL Inhibition Within and Among Bacterial Genera

We found no evidence of systematic variation among bacterial genera in their ability to inhibit multiple *Bd*GPL isolates. In our data, the principal source of variance in inhibition was among bacterial strains, with the number of strains demonstrating broad-spectrum *facilitation* of *Bd*GPL being roughly equal to the number exhibiting broad-scale *inhibition* of the pathogen. These data support previous work

suggesting *B. dendrobatidis* inhibition capability is distributed widely across bacterial genera (Antwis et al 2015; Becker et al 2015; Bletz et al 2017a); several strains demonstrated at least weak inhibition for all 10 *Bd*GPLs but were spread across multiple genera with no clear pattern. That there is clear functional redundancy among genera in this host-protective trait suggests it is not prudent to focus on any one genus in the search for beneficial probiotics (Becker et al 2015), as highly divergent microbial communities can still possess similar functional traits (e.g. Bletz et al 2016; 2017b).

We identified one *Bd*GPL isolate that was significantly prone to inhibition (08MG04) and a further two isolates that demonstrated strong resistance to inhibition (i.e. facilitated growth; AUL2 and JEL423). The phenomenon of *Bd*GPL growth facilitation has been described previously for single pathogen isolates (Bell et al 2013; Becker et al 2015), but crucially our results suggest that a bacterial strain's ability to facilitate the growth of *B. dendrobatidis* extends across a broad suite of pathogen isolates. Thus, facilitation of *B. dendrobatidis* growth is not simply a rare phenomenon arising from specific *Bd*GPL/bacterial combinations, and different *Bd*GPL isolates may differ systematically in their growth rates when exposed to bacterial filtrates (see also Muletz-Wolz et al 2017). It is unclear why some bacterial strains facilitate *B. dendrobatidis* growth, but one likely explanation is that certain bacterial metabolites can act as growth substrates or facilitators for fungi (Garbaye 1994; Hardoim et al 2015). In addition, different bacterial metabolites may alter the abiotic environment (e.g. pH) to confer different growth rates (Romanowski et al 2011) or hormesis may occur whereby the growth of *B. dendrobatidis* is facilitated at low or intermediate concentrations of particular bacterial products (Bell et al 2013).

Further research is required to determine whether a *Bd*GPL isolates' susceptibility to inhibition or facilitation correlates with virulence, and how genotypic traits associated with the pathogen map on to inhibition profiles and taxonomic traits of bacteria. It would also be valuable to further explore the effects of co-culturing bacteria with *B. dendrobatidis* prior to inhibition challenges, which may influence anti-*B. dendrobatidis* capabilities (Becker et al 2015). In particular, *B. dendrobatidis* isolates that elicit particularly strong metabolites from bacteria (i.e. *B. dendrobatidis* isolates that are readily inhibited) could be used to prime probiotic bacteria to make these more effective at inhibiting other more resistant *B. dendrobatidis* isolates, such as AUL2 and JEL423 in this study.

Consortium-Based Approaches to Combatting Fungal Pathogens

Our results revealed that the relationship between taxonomic diversity of a probiotic consortium and its ability to inhibit *B. dendrobatidis* growth was not consistent across *B. dendrobatidis* isolates. Multi-genus consortia outperformed both single-genus consortia and single bacterial strains in *B. dendrobatidis* inhibition, and were far more likely to produce strong inhibition of 50% or greater, but this is true for only for two of the three pathogen variants. Previous work has demonstrated a link between consortium species richness and *B. dendrobatidis* inhibition but only for a single pathogen isolate (Loudon et al 2014; Piova-Scott et al 2016). Our data suggest that this pattern may not be general, with marked variation among pathogen isolates in their susceptibility to multi-genus consortia.

That said, the general relationship (for two of the three pathogen variants) between inhibition and consortium diversity was in the expected direction; low community relatedness (i.e. high community dissimilarity) and high species richness both increase the resistance of a bacterial community to pathogenic 'invaders' (e.g. Jousset et al 2011; Eisenhauer et al 2012, 2013). That multi-genus consortia can provide superior inhibition for some pathogen variants is suggestive of synergistic effects, whereby the combined pool of metabolites from multiple bacteria inhibits *B. dendrobatidis* more strongly than the individual strains (Loudon et al. 2014). Superior inhibition from consortia, rather than single strains, may arise as a by-product of the interference competition over resources created by co-culture (Scheuring & Yu 2012). Bacteria that are weak inhibitors when used individually (as in this study) could increase the overall inhibitory power of a consortium by creating a competitive environment that favours greater production of anti-fungal compounds.

We found that one of the three *B. dendrobatidis* isolates (*Bd*GPL SFBC019) was not susceptible to inhibition from more diverse consortia as exhibited the other two pathogen variants (*Bd*CAPE TF5a1 and *Bd*GPL MODS 28.1). That *B. dendrobatidis* isolate can alter the strength of the relationship between consortium diversity and inhibition is a highly novel finding. *Bd*GPL SFBC019 appears largely resistant to inhibition irrespective of whether individual bacteria or consortia are used, with individual bacterial inhibition scores that were often negative (Fig. 3). This suggests resistance to inhibition from single strains may not necessarily be overcome by the putative synergistic effects from co-culturing bacteria, in the same way that total microbial communities (along with other anti-*B. dendrobatidis* factors associated with the skin) of amphibians may not always be resistant to particular variants of the pathogen (Antwis et al 2017). The underlying cause for this variation is unclear as our data

suggests this variation in consortia-based inhibition does not appear to correlate with *B. dendrobatidis* lineage. In addition, the results of the single strain challenges with 10 *Bd*GPL isolates showed all four isolates from one locality in the UK ("CORN" isolates; Table 1; Fig. 2) showed similar levels of inhibition across all bacterial strains, whereas the two isolates from the same locality in France ("AUL" isolates; Table 1; Fig. 2) exhibited markedly different inhibition profiles. This suggests even pathogen isolates collected from the same host species and locality have the potential to exhibit markedly different responses to bacterial probiotics. More work is required to determine the relative inhibition profiles of multiple *B. dendrobatidis* isolates challenged with single- and multi-bacteria probiotics across a spectrum of diversity, and to determine the mechanisms driving the responses of *B. dend*robatidis variants to these.

In the study presented here, some metabolites (and other bacterial products) will have been carried over from bacterial strains whilst constructing single and multi-species consortia, and it is also possible that after 12 hours of co-culture, the proportions of bacteria in the multi-species consortia were not equal. Thus, it would be beneficial to determine how inhibition profiles of mixed-species consortia alter over time and whether this can be optimised for the mitigation of wildlife disease. Similarly, understanding the response of the host microbiome to inoculation by probiotics, and concurrent factors that determine the longevity of probiotics on the skin of amphibians, would provide significant steps forward in developing effective treatments.

Conclusion

Our work has highlighted that different isolates of a lethal wildlife pathogen can vary in their susceptibility to probiotic bacteria, meaning we cannot expect probiotic effectiveness to be uniform across the genetic or phenotypic landscape of the pathogen. That said, higher diversity (richness and taxonomic) of probiotic consortia may provide greater protective capabilities against pathogens than individual bacteria, although some *B. dendrobatidis* isolates may be largely resistant to the majority of bacterial probiotics, and using bacterial consortia may not overcome this. These patterns are informative with respect to potential strategies for the application of bacterial probiotics to mitigate *B. dendrobatidis* and other wildlife pathogens. Conservationists might not always know which particular *B. dendrobatidis* variant is infecting a local population, preventing targeted application of known strong

inhibitors for that variant (Muletz-Wolz et al 2017), and both time and expense may prevent the establishment of such a database *de novo* if a probiotic intervention is required rapidly. Therefore, we must employ strategies that maximise the chance of successful inhibition in the absence of perfect knowledge of the pathogen. Although multi-genus consortia did not always outperform single-genus consortia or single bacteria strains, our data did reveal that these consortia have the highest probability of 'strong' inhibition of >50% if applied 'naively' without knowledge of the pathogen variant. This finding is important; human-mediated spread of *B. dendrobatidis* through the amphibian trade (Fisher & Garner 2007) means we cannot assume that local populations will be exposed to only one pathogenic variant. Future work will expand this study to test multi-genus consortia against a broader range of pathogen isolates to determine the generality of this pattern. It would be particularly interesting to combine whole-genome sequencing of the pathogen with inhibition data from single bacterial strains and consortia to assess whether closely related pathogen isolates are more likely to show similar responses, or lack thereof, to bacterial consortia. Despite the potential 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 quantifying how applicable inhibition measures derived *in vitro* are to real-world scenarios. Additionally, though we tend to treat bacterial inhibition scores as fixed traits, this ignores the ability of genetic recombination among *B. dendrobatidis* lineages to modify the relationship between bacterial metabolites and pathogen growth rates. Even the application of probiotics themselves may represent a strong selective pressure favouring genetic variants of *B. dendrobatidis* that lack susceptibility to those probiotics. Although several trials have demonstrated the potential for probiotic prophylaxis against *B. dendrobatidis*, we still lack the requisite data to measure selection caused by those trials on the pathogen. *In vitro* experimental evolution assays between pathogen and bacteria may prove the most powerful means for detecting such patterns.

ACKNOWLEDGEMENTS

This study was funded by a North-West University Postdoctoral Research Fellowship and a University of Salford Research Pump Priming Fund awarded to REA. XAH was funded by an Institute of Zoology

Research Fellowship. The authors would like to thank Prof Richard Preziosi and Prof Trenton Garner for additional provision of consumables, and Prof Ché Weldon, Prof Trenton Garner and Prof Matthew Fisher for access to *Batrachochytrium dendrobatidis* isolates used in this study.

DATA ACCESSIBILITY

All code and data to reproduce the results in this paper will be uploaded to FigShare upon publication at DOI 10.6084/m9.figshare.5633821

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.

REFERENCES

Antwis, R.E., Preziosi, R.F., Harrison, X.A., Garner, T.W. (2015). Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of Batrachochytrium dendrobatidis. Applied and Environmental Microbiology, 81, 3706-3711.

Antwis, R.E., Weldon, C. (2017) Amphibian skin defences show variation in ability to inhibit growth of *Batrachochytrium dendrobatidis* isolates from the Global Panzootic Lineage. Microbiology, doi: 10.1099/mic.0.000570.

Bates, D., Maechler, M., Bolker, B., Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67, 1-48. doi:10.18637/jss.v067.i01.

Becker, M.H., Walke, J.B., Murrill, L., Woodhams, D.C., Reinert, L.K., Rollins-Smith, L.A., et al. (2015). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen Batrachochytrium dendrobatidis. Molecular Ecology, 24, 1628-1641.

Bell, S.C., Alford, R.A., Garland, S., Padilla, G., Thomas, A.D. (2013). Screening bacterial metabolites for inhibitory effects against Batrachochytrium dendrobatidis using a spectrophotometric assay. Diseases of Aquatic Organisms, 103, 77-85.

Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P. et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. Ecology Letters, 16, 807-20.

Bletz, M.C., Goedbloed, D.J., Sanchez, E., Reinhardt, T., Tebbe, C.C., Bhuju, S., et al. (2016). Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. Nature Communications, 7.

Bletz, M.C., Myers, J., Woodhams, D.C., Rabemananjara, F.C.E., Rakotonirina, A., Weldon, C., et al. (2017a) Estimating Herd Immunity to Amphibian Chytridiomycosis in Madagascar Based on the Defensive Function of Amphibian Skin Bacteria. Front. Microbiol, 8, 1751. doi: 10.3389/fmicb.2017.01751

Bletz, M. C., Perl, R. G. B., Bobowski, B. T. C., Japke, L. M., Tebbe, C. C., Dohrmann, A. B. (2017b) Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted Bd-inhibitory function. ISME J, 11, 1521-1534. doi:10.1038/ismej.2017.41

497 Charif, D., & Lobry, J. R. (2007). SeqinR 1.0-2: a contributed package to the R project for statistical
498 computing devoted to biological sequences retrieval and analysis. *Structural approaches to sequence*
499 *evolution*, 207-232.

500

501 de Jong, M.D., Hien, T.T. (2006). Avian influenza A (H5N1). Journal of Clinical Virology, 35, 2-13.

502

503 Dillon, R.J., Vennard, C.T., Buckling, A., Charnley, A.K. (2005). Diversity of locust gut bacteria
504 protects against pathogen invasion. Ecology Letters, 8, 1291-8.

505

506 Doddington, B.J., Bosch, J., Oliver, J.A., Grassly, N.C., Garcia, G., Schmidt, B.R., et al. (2013),
507 Context-dependent amphibian host population response to an invading pathogen. Ecology, 94, 1795-
508 1804.

509

510 Eisenhauer, N., Scheu, S., Jousset, A. (2012). Bacterial diversity stabilizes community productivity.
511 PloS one, 7, e34517.

512

513 Eisenhauer, N., Schulz, W., Scheu, S., Jousset, A., Pfrender, M. (2013). Niche dimensionality links
514 biodiversity and invasibility of microbial communities. Functional Ecology, 27, 282-8.

515

516 Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W., Balloux, F., Clare, F., et al. (2011) Multiple
517 emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent
518 recombinant lineage. Proceedings of the National Academy of Sciences of the United States of
519 America, 108, 18732-6.

520

521 Farrer, R.A., Henk, D.A., Garner, T.W., Balloux, F., Woodhams, D.C., Fisher, M.C. (2013).
522 Chromosomal copy number variation, selection and uneven rates of recombination reveal cryptic
523 genome diversity linked to pathogenicity. PLoS Genetics, 9, p. e1003703.

Fisher, M.C., Garner, T.W.J. (2007) The relationship between the emergence of Batrachochytrium dendrobatidis, the international trade in amphibians and introduced amphibian species. Fungal Biology Reviews, 21, 2-9.

Fisher, M.C., Bosch, J., Yin, Z., Stead, D.A., Walker, J., Selway, L., et al. (2009) Proteomic and phenotypic profiling of the amphibian pathogen Batrachochytrium dendrobatidis shows that genotype is linked to virulence. Molecular ecology, 18, 415-29.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. Nature Reviews, 484, 186-194.

Garbaye, J. (1994). Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytologist, 128, 197-210.

Garner, T.W., Schmidt, B.R., Martel, A., Pasmans, F., Muths, E., Cunningham, A.A., et al. (2016). Mitigating amphibian chytridiomycoses in nature. Phil. Trans. R. Soc. B, 371, 20160207.

Geweke, J. (1992) Evaluating the accuracy of sampling-based approaches to calculating posterior moments. In *Bayesian Statistics 4* (ed JM Bernado, JO Berger, AP Dawid and AFM Smith). Clarendon Press, Oxford, UK.

Goka, K., Yokoyama, J., Une, Y., Kuroki, T., Suzuki, K., Nakahara, M., et al. (2009). Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. Molecular Ecology, 18, 4757-74.

Hadfield, J.D. (2010). MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package. Journal of Statistical Software, 33(2), 1-22. URL: http://www.jstatsoft.org/v33/i02/.

Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., et al. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiology and Molecular Biology Reviews, 79, 293-320. doi:10.1128/MMBR.00050-14.

Harris, R.N., Lauer, A., Simon, M.A., Banning, J.L., Alford, R.A. (2009). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. Diseases of Aquatic Organisms, 83, 11-16.

Hoyt, J.R., Cheng, T.L., Langwig, K.E., Hee, M.M., Frick, W.F., Kilpatrick, A.M. (2015). Bacteria isolated from bats inhibit the growth of Pseudogymnoascus destructans, the causative agent of white-nose syndrome. PLoS One, 10, e0121329.

Jani, A.J., Briggs, C.J. (2014). The pathogen Batrachochytrium dendrobatidis disturbs the frog skin microbiome during a natural epidemic and experimental infection. Proceedings of the National Academy of Sciences of the United States of America, 111, E5049-58.

Jenkinson, T.S., Betancourt Román, C.M., Lambertini, C., Valencia-Aguilar, A., Rodriguez, D., Nunes-de-Almeida, C.H., et al. (2016) Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. Molecular Ecology, 25, 2978–2996.

Jousset, A., Schmid, B., Scheu, S., Eisenhauer, N. (2011). Genotypic richness and dissimilarity opposingly affect ecosystem functioning. Ecology Letters, 14, 537-45.

Kaiser, K., Pollinger, J. (2012) Batrachochytrium dendrobatidis Shows High Genetic Diversity and Ecological Niche Specificity among Haplotypes in the Maya Mountains of Belize. PLoS ONE, 7, e32113. doi:10.1371/journal.pone.0032113

Kueneman, J.G., Woodhams, D.C., Harris, R., Archer, H.M., Knight, R., McKenzie, V.J. (2016) Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. Proc. R. Soc. B, 283, 1839.

Loudon, A. H., Holland, J. A., Umile, T. P., Burzynski, E. A., Minbiole, K. P. C., & Harris, R. N. (2014). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. Frontiers in Microbiology, 5. doi:10.3389/fmicb.2014.00441

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., et al. (2013). Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. Proceedings of the National Academy of Sciences, 110, 15325-15329.

Matos, A., Kerkhof, L., Garland, J.L. (2005). Effects of microbial community diversity on the survival of Pseudomonas aeruginosa in the wheat rhizosphere. Microbial Ecology, 49, 257-64.

McCallum, H. (2012) Disease and the dynamics of extinction. Philos Trans R Soc Lond B Biol Sci 367: 2828–2839. doi: 10.1098/rstb.2012.0224. pmid:22966138

Morgan, J.A., Vredenburg, V.T., Rachowicz, L.J., Knapp, R.A., Stice, M.J., Tunstall, T., et al. (2007). Population genetics of the frog-killing fungus *Batrachochytrium dendrobatidis*. Proceedings of the National Academy of Sciences of the United States of America, 104, 13845-50.

604 Muletz, C.R., Myers, J.M., Domangue, R.J., Herrick, J.B., Harris, R.N. (2012). Soil bioaugmentation
605 with amphibian cutaneous bacteria protects amphibian hosts from infection by Batrachochytrium
606 dendrobatidis. Biological Conservation, 152, 119-126.

607

608 Olson, D.H., Aanensen, D.M., Ronnenberg, K.L., Powell, C.I., Walker, S.F., Bielby, J., et al. (2013).
609 Mapping the global emergence of Batrachochytrium dendrobatidis, the amphibian chytrid fungus. PloS
610 one, 8, e56802.

611

612 Piovia-Scott, J., Rejmanek, D., Woodhams, D. C., Worth, S. J., Kenny, H., McKenzie, V., et al. (2017).
613 Greater Species Richness of Bacterial Skin Symbionts Better Suppresses the Amphibian Fungal
614 Pathogen Batrachochytrium Dendrobatidis. Microbiology Ecology, DOI 10.1007/s00248-016-0916-4.

615

616 Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The
617 SILVA ribosomal RNA gene database project: improved data processing and web-based tools.
618 *Nucl. Acids Res. 41 (D1): D590-D596.*

619

620 R Core Team. (2016). R: A language and environment for statistical computing. R Foundation for
621 Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

622

623 Rebollar, E.A., Antwis, R.E., Becker, M.H., Belden, L.K., Bletz, M.C., Brucker, R.M., et al. (2016).
624 Using "Omics" and Integrated Multi-Omics Approaches to Guide Probiotic Selection to Mitigate
625 Chytridiomycosis and Other Emerging Infectious Diseases. Frontiers in Microbiology, 7, 68.

626

627 Rodriguez, D., Becker, C. G., Pupin, N.C., Haddad, C.F.B., Zamudio, K. R. (2014). Long-term
628 endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of
629 Brazil. Molecular Ecology, 23, 774-87.

630

631 Romanowski, K., Zaborin, A., Fernandez, H., Poroyko, V., Valuckaite, V., Gerdes, S., et al. (2011).
632 Prevention of siderophore- mediated gut-derived sepsis due to P. aeruginosa can be achieved without
633 iron provision by maintaining local phosphate abundance: role of pH. BMC Microbiology, 11: 212

634

635 Rosenblum, E.B., James, T.Y., Zamudio, K.R., Poorten, T.J., Ilut, D., Rodriguez, D., et al. (2013).
636 Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data.
637 Proceedings of the National Academy of Sciences of the United States of America, 110, 9385-90.

638

639 Scheuring, I., Yu, D.W. (2012). How to assemble a beneficial microbiome in three easy steps. Ecology
640 Letters, 15, 1300-7.

641

642 Schock, D.M., Bollinger, T.K., Collins, J.P. (2009). Mortality rates differ among amphibian populations
643 exposed to three strains of a lethal ranavirus. EcoHealth, 6, 438-48.

644

645 Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., et al. (2012)
646 Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog
647 trade. Molecular Ecology, 21, 5162-77.

648

649 Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K.R., et al. (2007) Spread of
650 chytridiomycosis has caused the rapid global decline and extinction of frogs. Ecohealth 4: 125–134.

651

652 Sleeman, J.M. (2013). Has the time come for big science in wildlife health? EcoHealth, 10, 335-338.

653

654 Tompkins, D.M., Carver, S., Jones, M.E., Krkošek, M., Skerratt, L.F. (2015). Emerging infectious
655 diseases of wildlife: a critical perspective. Trends in Parasitology, 31, 149-159.

656

van Elsas, J.D., Chiurazzi, M., Mallon, C.A., Elhottova, D., Kristufek, V., Salles, J.F. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. Proceedings of the National Academy of Sciences of the United States of America, 109, 1159-64.

Walke, J.B., Becker, M.H., Loftus, S.C., House, L.L., Teotonio, T.L., Minbiole, K.P. et al. (2015). Community Structure and Function of Amphibian Skin Microbes: An Experiment with Bullfrogs Exposed to a Chytrid Fungus. PloS one, 10, e0139848.

Woodhams, D.C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS One*, *9*(4), p.e96375.

Yasumiba, K., Bell, S., Alford, R. (2016). Cell density effects of frog skin bacteria on their capacity to inhibit growth of the chytrid fungus, *Batrachochytrium dendrobatidis*. Microbial Ecology, 71, 124-30. doi:10.1007/s00248-015-0701-9

683 **TABLE LEGENDS**

684 **Table 1.** *Batrachochytrium dendrobatidis* isolates used in the study.

685

686 **Table 2.** Mean Proportion of 10 *Bd*GPL isolates for which at least weak inhibitory capability was
687 observed, averaged over all bacterial strains in a genus. 95% CI: 95% confidence intervals from an
688 overdispersion-corrected Binomial GLM.

689

690

691 **FIGURE LEGENDS**

692 **Figure 1. Inhibition scores of 54 bacterial strains from 10 genera when tested against 10**
693 ***Bd*GPL isolates.** A positive value represents inhibition of *B. dendrobatidis* growth and a negative
694 value indicates enhanced growth of *B. dendrobatidis*. Estimates are derived from a Bayesian mixed
695 effects model with bacterial strain nested within genus, and *Bd*GPL isolate fitted as random effects.
696 Points are conditional modes of the individual bacterial strain random effects, marginalised with
697 respect to *Bd*GPL isolate. Error bars are 95% credible intervals. Bacterial strains from the same genus
698 are denoted by the same colour.

699

700 **Figure 2. Heat map of inhibition across all 54 bacterial strains and all 10 *B. dendrobatidis***
701 **isolates.**

702 Bacterial strains have been clustered according to phylogeny and *B. dendrobatidis* isolates have been
703 clustered according to their similarity in inhibition profiles (dendrograms in left and top margins,
704 respectively). Inhibition scores have been z-transformed across *B. dendrobatidis* isolates (rows) for
705 each particular bacterial strain. Bacterial row names include both genus and strain ID. Blue indicates
706 low inhibition, through to red, which indicates high inhibition.

707

708

Figure 3. Inhibition scores for Single-Genus (SG) and Multi-Genus (MG) Consortia across three *B. dendrobatidis* isolates (*Bd*GPL MODS28.1, *Bd*GPL SFBC019 and *Bd*CAPE TF5a1). A positive value represents inhibition of *B. dendrobatidis* growth and a negative value indicates enhanced growth of *B. dendrobatidis*. Points have been jittered for display purposes.

Figure 4. Relationship between mean genetic distance among consortium members and *B. dendrobatidis* inhibition score.

We detected a significant positive relationship between genetic distance and inhibition percentage for BdCAPE TF5a1 and BdGPL MODS28.1 but not BdGPL SFBC019. Fitted lines and shaded areas are mean and 95% confidence intervals from a linear model fit.

Figure 5. Randomisation results examining the relative efficacy of different probiotic strategies. (A) the probability of Multi-Genus Consortia (MGC) yielding higher inhibition compared to Single-Genus Consortia (SGC) or a single bacterial strain (Single); (B) the probability of MGC, SGC or single bacteria yielding inhibition > 50% when applied to each of three *B. dendrobatidis* isolates; (C) The probability of an individual consortium type yielding >50% inhibition when applied to two randomly chosen *B. dendrobatidis* isolates in series.

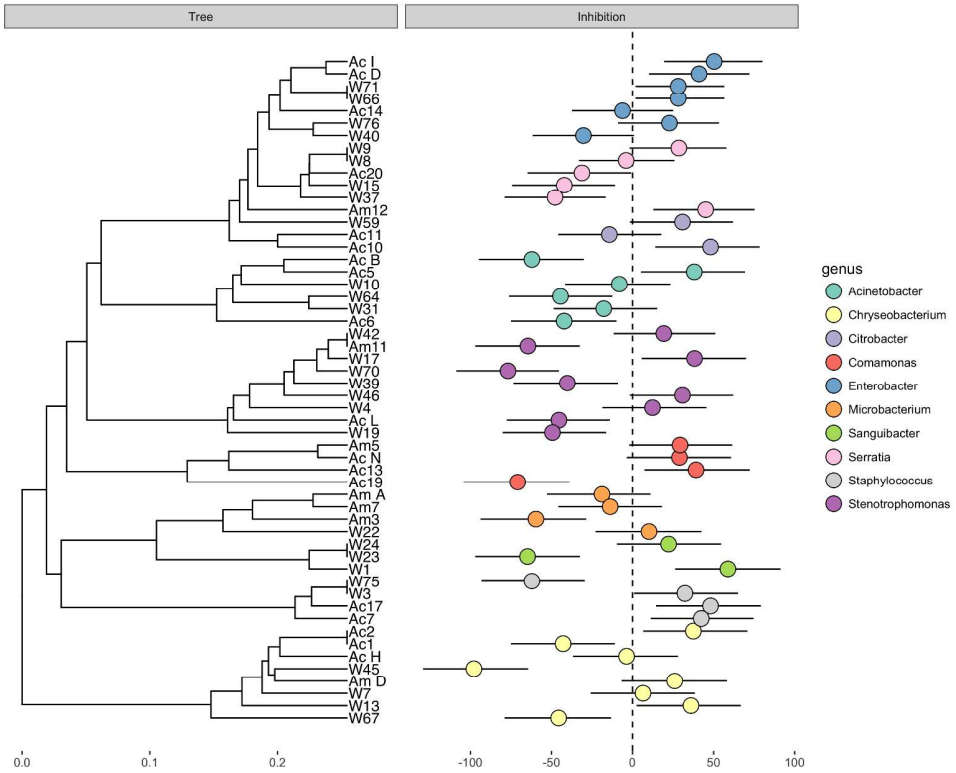
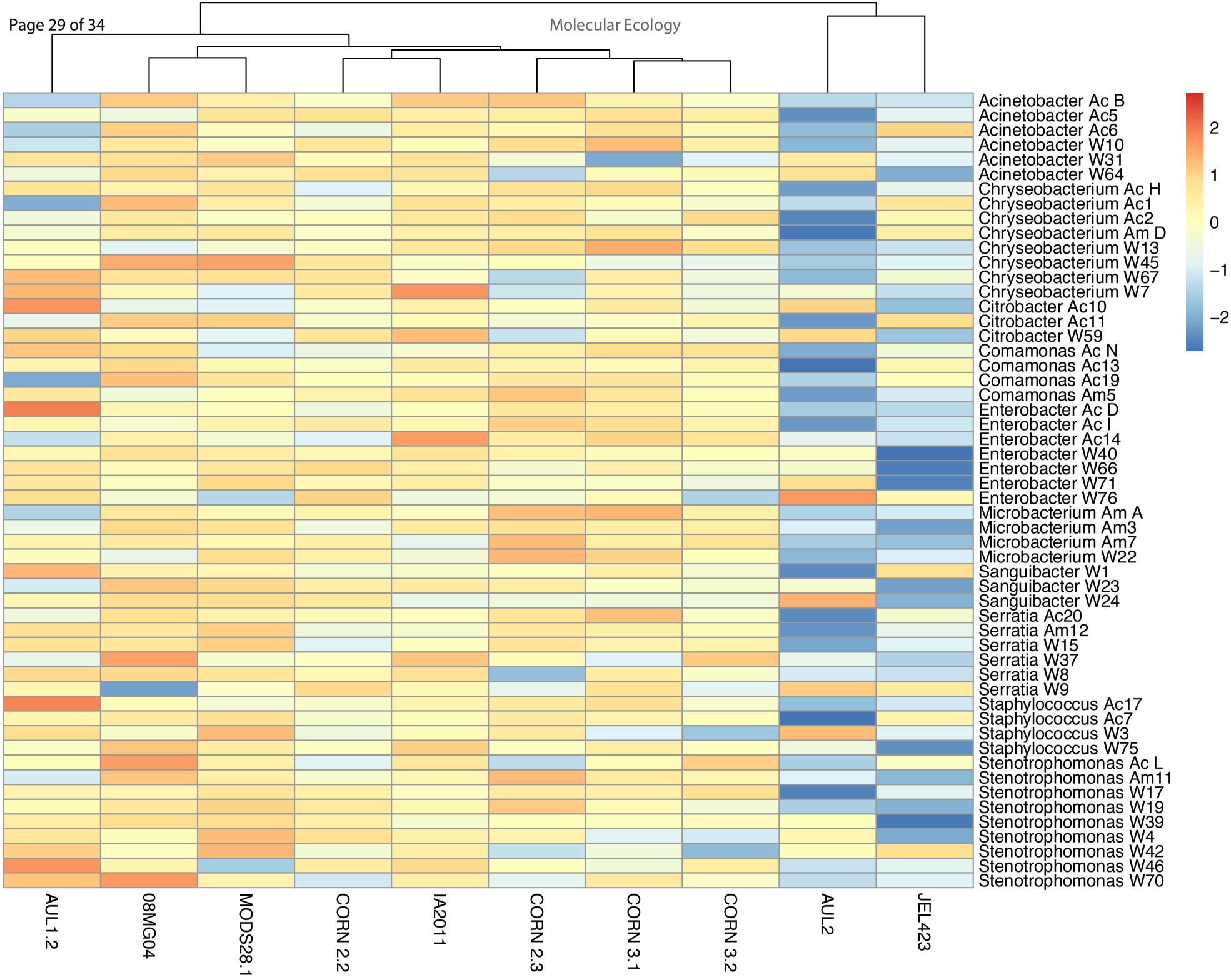


Figure 1. Inhibition scores of 54 bacterial strains from 10 genera when tested against 10 BdGPL isolates. A positive value represents inhibition of B. dendrobatidis growth and a negative value indicates enhanced growth of B. dendrobatidis. Estimates are derived from a Bayesian mixed effects model with bacterial strain nested within genus, and BdGPL isolate fitted as random effects. Points are conditional modes of the individual bacterial strain random effects, marginalised with respect to BdGPL isolate. Error bars are 95% credible intervals. Bacterial strains from the same genus are denoted by the same colour.

1041x833mm (72 x 72 DPI)


Acinetobacter Ac B
Acinetobacter Ac5
Acinetobacter Ac6
Acinetobacter W10
Acinetobacter W31
Acinetobacter W64
Chryseobacterium Ac H
Chryseobacterium Ac1
Chryseobacterium Ac2
Chryseobacterium Am D
Chryseobacterium W13
Chryseobacterium W45
Chryseobacterium W67
Chryseobacterium W7
Citrobacter Ac10
Citrobacter Ac11
Citrobacter W59
Comamonas Ac N
Comamonas Ac13
Comamonas Ac19
Comamonas Am5
Enterobacter Ac D
Enterobacter Ac I
Enterobacter Ac14
Enterobacter W40
Enterobacter W66
Enterobacter W71
Enterobacter W76
Microbacterium Am A
Microbacterium Am3
Microbacterium Am7
Microbacterium W22
Sanguibacter W1
Sanguibacter W23
Sanguibacter W24
Serratia Ac20
Serratia Am12
Serratia W15
Serratia W37
Serratia W8
Serratia W9
Staphylococcus Ac17
Staphylococcus Ac7
Staphylococcus W3
Staphylococcus W75
Stenotrophomonas Ac L
Stenotrophomonas Am11
Stenotrophomonas W17
Stenotrophomonas W19
Stenotrophomonas W39
Stenotrophomonas W4
Stenotrophomonas W42
Stenotrophomonas W46
Stenotrophomonas W70
2
1
0
−1
−2
AUL1.2
08MG04
MODS28.1
CORN 2.2
IA2011
CORN 2.3
CORN 3.1
CORN 3.2
AUL2
JEL423

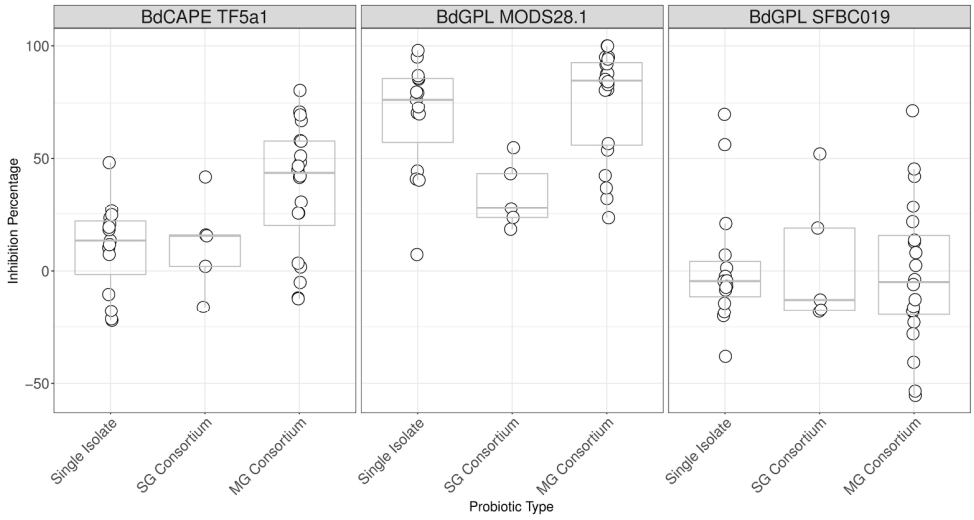


Figure 3. Inhibition scores for Single-Genus (SG) and Multi-Genus (MG) Consortia across three B. dendrobatidis isolates (BdGPL MODS28.1, BdGPL SFBC019 and BdCAPE TF5a1). A positive value represents inhibition of B. dendrobatidis growth and a negative value indicates enhanced growth of B. dendrobatidis. Points have been jittered for display purposes.

203x108mm (300 x 300 DPI)

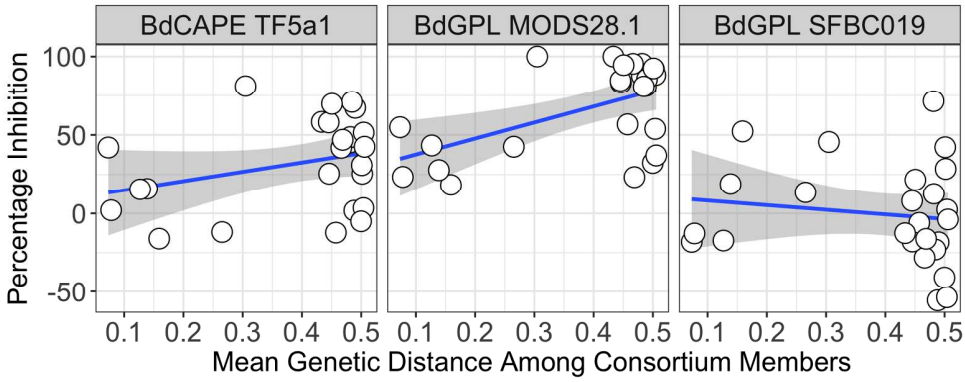


Figure 4. Relationship between mean genetic distance among consortium members and B. dendrobatidis inhibition score.
We detected a significant positive relationship between genetic distance and inhibition percentage for BdCAPE TF5a1 and BdGPL MODS28.1 but not BdGPL SFBC019. Fitted lines and shaded areas are mean and 95% confidence intervals from a linear model fit.

793x317mm (72 x 72 DPI)

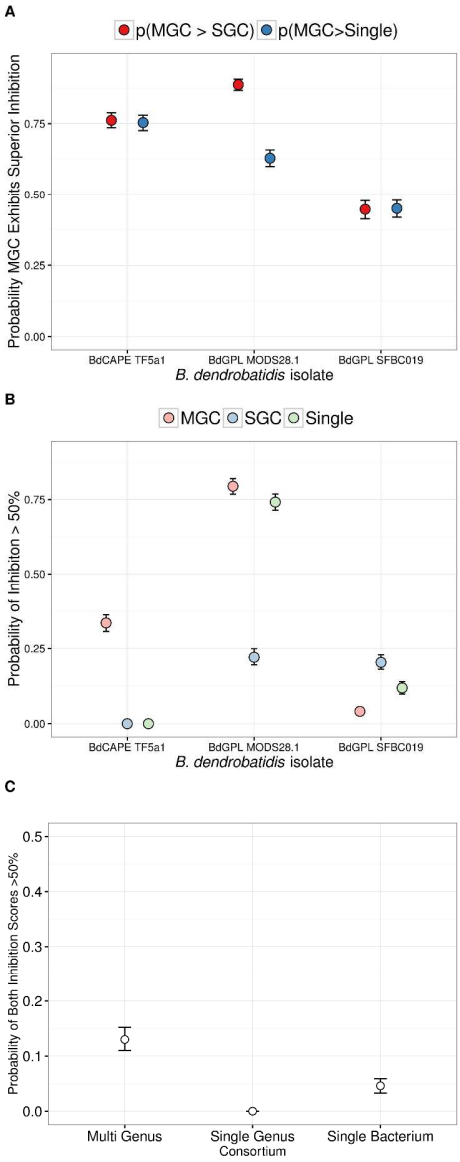


Figure 5. Randomisation results examining the relative efficacy of different probiotic strategies. (A) the probability of Multi-Genus Consortia (MGC) yielding higher inhibition compared to Single-Genus Consortia (SGC) or a single bacterial strain (Single); (B) the probability of MGC, SGC or single bacteria yielding inhibition > 50% when applied to each of three B. dendrobatidis isolates; (C) The probability of an individual consortium type yielding >50% inhibition when applied to two randomly chosen B. dendrobatidis isolates in series.

381x952mm (300 x 300 DPI)

Table 1. *Batrachochytrium dendrobatidis* isolates used in the study.

Isolate	Lineage	Geographic	Host species	Collector	Year	Phylogeny screening	Consortium challenges
MG04	GPL	Silver Mine, Western Cape, South Africa	*Amietia fuscigula*	Trenton Garner	2010	X	
CORN2.2	GPL	Penhale Farm, Cornwall, UK	*Ichthyosaurus alpestris*	Trenton Garner	2012	X	
CORN2.3	GPL	Penhale Farm, Cornwall, UK	*Ichthyosaurus alpestris*	Trenton Garner	2012	X	
CORN3.1	GPL	Penhale Farm, Cornwall, UK	*Ichthyosaurus alpestris*	Trenton Garner	2012	X	
CORN3.2	GPL	Penhale Farm, Cornwall, UK	*Ichthyosaurus alpestris*	Trenton Garner	2012	X	
AUL1.2	GPL	Lac d'Aule, France	*Alytes obstetricans*	Matthew Fisher	2010	X	
AUL2	GPL	Lac d'Aule, France	*Alytes obstetricans*	Matthew Fisher	2010	X	
IA2011	GPL	Ibon Acherito, Spain	*Alytes obstetricans*	Matthew Fisher	2011	X	
MODS 28.1	GPL	Mont Olia, Sardinia	*Discoglossus sardus*	Trenton Garner	2010	X	
JEL423	GPL	Guabal, Panama	*Agalychnis lemur*	Joyce Longcore	2004	X	X
SFBC019	GPL	Sellafield, Cumbria, UK	*Epidalea calamita*	Peter Minting	2010		X
TF5a1	CAPE	Torrent des Ferrerets, Mallorca	*Alytes muletensis*	Matthew Fisher	2007		X

Table 2. Mean Proportion of 10 *Bd*GPL isolates for which at least weak inhibitory capability was observed, averaged over all bacterial strains in a genus. 95% CI: 95% confidence intervals from an overdispersion-corrected Binomial GLM.

Genus	**Number of Isolates**	**Mean Proportion *BdGPL* Inhibition**	**95% CI**
Acinetobacter	6	0.33	0.1-0.64
Chryseobacterium	8	0.50	0.25-0.75
Citrobacter	3	0.67	0.24-0.95
Comamonas	4	0.70	0.32-0.94
Enterobacter	7	0.73	0.44-0.92
Microbacterium	4	0.40	0.1-0.76
Sanguibacter	3	0.63	0.22-0.94
Serratia	6	0.47	0.19-0.76
Staphylococcus	4	0.73	0.35-0.96
Stenotrophomonas	9	0.49	0.25-0.73