Introduction

The world’s amphibians are facing a conservation crisis due to a variety of factors [1], with the newest and perhaps most insidious threat coming from an emerging global pathogen, the microscopic fungus Batrachochytrium dendrobatidis, or Bd [2]. This fungus is the etiological agent of chytridiomycosis, and has been implicated in many enigmatic declines worldwide [3,4,5]. In the Western Hemisphere, the disease has mainly affected populations in highland environments [6,7,8,9]. Species occurring in tropical Hemisphere, the disease has mainly affected populations in many enigmatic declines worldwide [3,4,5]. In the Western Hemisphere, the disease has mainly affected populations in highland environments [6,7,8,9]. Species occurring in tropical regions at middle and high elevations are thought to face a higher risk presumably because (1) Bd grows faster at the low temperatures characteristic of montane areas [10,11], and (2) the amphibian immune capacity may decrease at lower temperatures [7,12]. Despite the current focus on montane declines, a growing body of evidence suggests that Bd may also be widespread and abundant in lowland amphibian populations, for example, in Australia and Central America [11,13,14], yet amphibian declines are rarely observed in the lowlands [14,15].

Individuals, populations and species of amphibians are known to vary in their susceptibility to chytridiomycosis, but the causes of this variation are not well understood [16]. Some species consist of both resistant and susceptible populations [6]. Some species maintain remnant populations that persist in the presence of Bd [17,18], whereas others are known to be infected but show no signs of chytridiomycosis [5,19]. The allegedly well developed immune system of amphibians, which shows both adaptive and innate responses [7], is probably involved on the resistance to infection [20,21]. Amphibians may also benefit from symbiotic microbiota present on their skin that function as a barrier against pathogens, including Bd, which specifically targets amphibian skin [22,23,24].

Previous work has demonstrated that bacterial isolates from the skin of certain species of amphibians exhibit strong antifungal activity against Mortierella sp. [25] and Bd [22,26]. For example, the bacteria Janthinobacterium lividum and Lysobacter gummosus, produce the antimicrobial peptides violacein and 2,4-diacetylphloroglucinol respectively, which inhibit Bd growth in vitro [27,28]. Thus, variation in the cutaneous microbial community of amphibian skin may be a key factor in resistance to chytridiomycosis [26,28,29,30]. Conservation strategies focused on amphibians threatened by chytridiomycosis have mainly sought to prevent the spread of Bd to naive populations and establish ex situ assurance colonies, yet solving the problem may require prophylactic treatments and enabling populations to persist with the pathogen [31]. Since the discovery of cutaneous bacteria with strong antifungal properties, increasing effort has focused on probiotic therapies [25,30]. Perhaps the most promising approach to date is to use beneficial
symbiotic microorganisms, or their metabolic products, to increase the resistance to infection or disease through environment bioaugmentation or host therapy [16,30,31,32]. However, the majority of the studies had not considered the role of the environmental conditions on this host-pathogen interaction, since small variations could strongly affect anti-Bd activity of protective bacteria [33].

While most research on the immune-like properties of the cutaneous microbiota of amphibian skin has been conducted on temperate zone systems [32,34], the vast majority of amphibian diversity lies in the tropical realm, especially in South America [35]. If microbial communities on tropical amphibians differ from those on temperate hosts, then probiotic therapies optimized for these highly endangered tropical species are needed [36]. Furthermore, since bioaugmentation and other probiotic approaches would eventually require the anthropogenic introduction of bacteria into an environment, using locally obtained bacteria might minimize the risks associated with this procedure. Therefore, surveying the diversity and evaluating the anti-Bd action of microbial communities in the amphibian skin of Neotropical species could potentially expand the array of tools to help mitigate the impact of Bd.

One of the more severely impacted groups of amphibians are the montane harlequin toads of the genus, *Atelopus* (Anura: Bufonidae) with 80% of species listed as critically endangered [37]. In Colombia nearly all the 33 species of *Atelopus* have declined sharply, yet four lowland species are persisting at 0–600 masl [38]. For at least one species, *A. elegans* from Gorgona Island, we know that the pathogen has been present for at least five years without causing obvious disease or declines [39]. Among the various possible factors that may account for the persistence of *A. elegans* despite infection with Bd we hypothesize that cutaneous symbiotic bacteria may be a contributing factor to disease resistance in this population.

The aim of our study was to test whether the frog *A. elegans* harbors cutaneous bacteria capable of inhibiting Bd growth. We also compared the antifungal bacterial communities found in *A. elegans* with two other *Atelopus* species that persist in the lowlands without evidence of Bd infection. Thus, our null hypothesis is that bacteria isolated from the infected species (*A. elegans*) should exhibit stronger anti-Bd activity compared with bacteria from the other two toad species. Our long-term goal is to know whether these potentially beneficial strains could be used in bioaugmentation experiments or the metabolites they produce employed in host therapy to protect threatened Neotropical species.

### Materials and Methods

**Ethics statement**

Procedures for capture and handling of live animals in the field were approved by the Colombian National Parks authority and the Ministry of the Environment, under permits DTSO 019-09, DTSO 001-09 and N° 10-07032012.

**Study species**

Cutaneous bacterial microbiota was sampled from three latitudinally separated species occurring in coastal forests of Colombia. *Atelopus aff. limosus*, probably an undescribed species, occurs near the municipality of Capurganá (8° 37’N, 77° 22’W, 150 masl), close to the border between Panama and Colombia; *Atelopus spurrelli*, considered as Vulnerable (VU) by the IUCN [40], was sampled near the municipality of Arusí (5°30’N, 77°31’W, 90 masl) on the Pacific coast; and *A. elegans*, Critically Endangered (CR), was sampled in the insular Gorgona National Park located 56 Km off the Pacific coast of Colombia (2°47’–3°6’N, 78°6’–78°18’ W, between 6–115 masl). *A. elegans* had tested positive (17%, 15 out of 78 individuals) for Bd at the time we conducted this study [39]. We sampled 80 individuals of *A. spurrelli* and 82 of *A. aff. limosus* and detected no infected individuals. We thus concluded that Bd might be either absent or rare in those populations.

**Bacterial isolation**

To obtain bacterial isolates from toads’ skin, samples were collected from five *A. spurrelli*, eight *Atelopus aff. limosus* and seven *A. elegans* adults. Toads were manipulated using fresh disposable nitrile gloves and rinsed twice in sterile dechlorinated water to remove transient bacteria [25]. Individuals were swabbed on their left, right and ventral surfaces, hindlimbs and interdigital membranes, using a sterile cotton swab. Swabs were preserved in 2 mL cryovials containing 1 mL DS solution, a weak salt solution resembling pond water [41]. All swab samples were refrigerated within 24 h and processed within 48 hours after sampling. To isolate pure colonies, serial dilutions were performed until 1 × 10⁻⁵. To recover the highest possible number of bacterial morphotypes, each dilution was plated in R2A media in duplicate and incubated at 23°C for two days. Bacterial morphotypes were defined according to the macroscopic characteristics of the obtained colonies (i.e. color, form, elevation and margin). Single colonies of each bacterial morphotype were streaked on fresh nutritive agar plates until pure cultures were obtained. Each isolate was cryopreserved in nutritive broth with 30% glycerol at −80°C.

**Batrachochytrium dendrobatis growth inhibition assays**

To test for anti-Bd activity in bacteria isolated from frogs’ skin, we used growth inhibition assays [22]. At the time of the assays, no Colombian Bd strains were available, so we decided to use Bd strain JEL 423 (University of Maine, Orono, USA) isolated from *Phyllomedusa lemur* in lowland forests of neighboring Panama. Bd was grown in TGH media (10 g tryptone, 10 g agar, 4 g gelatine hydrolysate, 1000 mL distilled water) for three days at 23°C until maximal zoospore production was observed. Bd was then harvested from three Petri dishes and transferred to a sterile tube containing 16 mL of sterile water in order to obtain a solution with a high concentration of zoospores. Plates with 25 mL of TGH media were coated with 1 mL of the zoospore suspension, and plates were allowed to dry until most of the solution had diffused into the agar. Unknown or “query” bacteria from active isolated cultures were streaked in a line across one side of a Petri dish. *Escherichia coli* (strain DH5α) was streaked as a negative control in a parallel line on the opposite side of the Petri dish; this control streak was included to observe the Bd growth around a bacterial species that shows no inhibition activity on Bd growth. Each bacterial isolate was tested in triplicate and the plates were incubated at 23°C. On the third day of incubation, query bacteria were checked for signs of inhibition of Bd growth. For those isolates showing inhibition, photographs were taken and anti-Bd activity was estimated by using photometric techniques.

We found two kinds of evidence of anti-Bd activity in the tested cultures. First, we detected an inhibition zone around the query bacteria line. Second, we found strong variation in the density of Bd colonies growing throughout the culture medium outside the inhibition zone. To summarize both effects in a single measurement we took photographs of the cultures under similar lighting conditions with a dark background. Then, we estimated Bd growth by measuring the light (i.e. grey value) reflected at variable distances of the query bacteria line (Fig. 1). Data from the three plates were averaged in order to obtain the statistical unit of
To facilitate comparisons we re-expressed grey values as percentages of the maximum light intensity reflected by a single Bd colony within the same Petri dish. All photometric measurements were conducted on the software ImageJ [42] after distance and light calibration.

To quantify Bd growth inhibition we modeled Grey values (hereafter Bd growth) as a function of distance to the query bacteria by using spline models on JMP statistics software (JMP, Version 8. SAS Institute Inc., Cary, NC, 1998–2007). Spline models combine several polynomial functions of relatively low degree (often cubic) to fit piecewise several subsets of X-Y values. A Lambda parameter allows modifying the shape of the curve between a pure cubic function (Lambda = 0) and eventually a straight line, as Lambda diverges to infinity [43]. The functions are blend smoothly allowing both interpolation of Y-values as well as estimation of model residuals.

Identification of bacterial isolates

Bacterial strains were identified by sequencing the 16S ribosomal gene. One colony of each morphotype was re-suspended in 10 µL of distilled water in a 0.2 mL PCR tube and boiled for 7 min at 95°C. The solution was used directly in a PCR reaction with the universal eubacterial primers, 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACCTT-3’) [44]. Thermocycling parameters were: an initial denaturation of 3 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 52°C and 90 s at 72°C. A final
extension of 7 min at 72°C was performed to complete polymerization. PCR results were checked by electrophoresis in 1% agarose gels. Products were sent to Macrogen (Korea) for sequencing. DNA sequences were cleaned and assembled using Genero [45]. 16S sequences were then identified using BLASTn [46] against the complete GenBank nucleotide database (http://www.ncbi.nlm.nih.gov) and the Greengenes database (http://greengenes.lbl.gov), using default parameter settings in both cases.

**Results**

A total of 148 cultivable bacterial morphotypes were isolated, 40 from *Atelopus elegans*, 83 from *Atelopus aff. limosus*, and 23 from *A. spurrelli*. In antagonism assays, we observed anti-\(Bd\) activity in 16 of 40 (40%) bacterial morphotypes from *A. elegans*, 16 of 83 from *Atelopus aff. limosus* (19%), and six of 23 (26%) from *A. spurrelli*. We detected an inhibition zone around the query bacteria line, and also found strong variation in the density of \(Bd\) growing in the Petri dish outside the inhibition halo (Fig. 1).

Among all three hosts we identified 12 bacterial species with anti-\(Bd\) activity belonging to six genera: *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, *Comamonas*, *Chryseobacterium* and *Elisabethella* (Table 1). Toad species differed more in the composition of bacteria with anti-\(Bd\) activity (Anosim \(R=0.42, P<0.0001, 9999\) permutations) than in the whole bacterial communities (\(R=0.51, P<0.0001\)) as inferred from Bray-Curtis indices of dissimilarity: \(0.71 (0.59\) for all cultivable bacteria) between *Atelopus aff. limosus* and *A. spurrelli*, \(0.84 (0.69\) between *Atelopus aff. limosus* and *A. elegans*, and \(0.75 (0.71\) between *A. spurrelli* and *A. elegans*, ranging between 0 = identical and 1 = totally dissimilar. Two out of the three strains that exhibited the highest anti-\(Bd\) action, both tentatively assigned to *P. tolaasii*, were exclusive to *A. elegans*. The third one, tentatively assigned to *P. putida*, was isolated from *A. spurrelli* skins.

A generalized spline model was fitted to \(Bd\) growth as measured within the Petri dish at variable distances to query bacteria (Fig. 1). The regression residuals were re-analyzed by toad species and showed clearly that bacterial strains isolated from *A. elegans* caused greater \(Bd\) growth inhibition in *vitro* as compared to the other toad species (Fig. 2). To fit a spline model (\(Bd\) growth as a function of distance to the query bacteria) for each bacterial strain (Fig. 3) and interpolated \(Bd\) growth values at the first, second and third quartile of the growth function. Strains isolated from the skin of *A. elegans* showed higher anti-\(Bd\) activity (i.e. lower \(Bd\) growth; Manova, Frog, \(F=3.292, DF=2, P=0.0490\)) especially at the first and second quartiles of the growth curves (Manova, Frog \(\times\) Quartile interaction, Wilks’ lambda value = 0.619, \(DF=4, P=0.0024, N=38\) tests; Fig. 4).

**Discussion**

Our results demonstrated that 12 bacterial species isolated from three *Atelopus* species from the Colombian lowlands inhibit \(Bd\) growth. The composition of anti-\(Bd\) bacterial communities significantly varied between toad species or perhaps localities. The strongest anti-\(Bd\) activity was measured in bacteria isolated from *A. elegans*, the only species that tested positive for the pathogen. Our data suggest two evolutionary mechanisms behind the frog-pathogen-bacteria interaction. Of course, the proportion of bacteria shared among toad species is probably underestimated since we worked only with cultivable bacteria. In any case, bacterial communities may covary with distance or geographic conditions, it is important to look for candidate local strains with strong anti-\(Bd\) activity before considering bioaugmentation assays and eventual probiotic treatment of chytridiomycosis.

*Atelopus elegans*, the only species in our study that tested positive for \(Bd\), holds the skin bacteria with the strongest anti-\(Bd\) action. Although admittedly non-replicated, the pattern suggests that the species’ current bacterial community may have resulted from natural selection represented by \(Bd\) infection. If so, we would be witnessing a post-infection or post-decline event in *A. elegans*, where frogs and pathogen are now coexisting after a critical period of strong natural selection. Alternatively, the anti-\(Bd\) bacterial microbiota of the three toad species may represent an exaptation, a pre-existing (extended) phenotype that eventually subserves protection against \(Bd\) pathogenic infection. To discriminate between both the adaptation and exaptation scenarios, we need longitudinal (i.e. historical within the same population) data or geographic (i.e. between populations) comparisons on bacterial composition of infected and uninfected frogs. In the latter case, Woodhams et al. [26], have already showed that *Rana muscosa* from Conness in Yosemite National Park hosts a significant proportion of anti-\(Bd\) bacteria that inhibited \(Bd\) growth and persisted for six years in the presence of the chytrid fungus, whereas the Sixty Lake population was devastated by chytridiomycosis. For evolution to occur on skin microbiota, the ability to acquire or maintain certain bacteria should be heritable. Frog skins might vary in their habitability to different bacterial taxa due to their skin secretions or skin humidity; also, between species differences in habitat use may affect the probability of acquiring certain bacterial taxa. Both represent just speculative hypotheses that deserve rigorous testing.

We cannot rule out the possibility that high temperatures delay or reduce \(Bd\) growth in the studied *Atelopus* species, since they inhabit lowland forests of Colombian coasts with average annual temperatures around 27°C. High environmental temperatures can be directly involved with the growth control of the pathogen. Laboratory tests have shown that at temperatures higher than 25°C the pathogen either decreases zoospore production or dies [10]. Moreover, other studies showed that \(Bd\)-infected frogs exposed to warmer temperatures lived longer [50] and that the prevalence of infection was considerably lower [51]. In our case,
high temperatures may have interacted with species-specific microbiota in allowing the survival of *A. elegans* despite the infection by *Bd*. The effect of environmental conditions in shaping

the interaction between frogs and *Bd* has been already suggested [33].

Table 1. Prevalence of bacterial species isolated from the skin of *Atelopus aff. limosus, A. spurrelli* and *A. elegans*.

| Bacterial Isolates              | Atelopus aff. limosus | Atelopus spurrelli | Atelopus elegans |
|--------------------------------|-----------------------|-------------------|------------------|
| Acinetobacter baumanii         | 3                     | 2                 | 0                |
| Acinetobacter calcoaceticus    | 2                     | 1                 | 0                |
| Acinetobacter genomosp.        | 1                     | 0                 | 0                |
| Acinetobacter gyllenbergii     | 5*                    | 1*                | 0                |
| Acinetobacter venetianus       | 1                     | 0                 | 0                |
| Acinetobacter haemolyticus     | 0                     | 1*                | 0                |
| Acinetobacter junii            | 1                     | 0                 | 0                |
| Acinetobacter sp.              | 7*                    | 2*                | 3*               |
| Chryseobacterium sp.           | 5*                    | 0                 | 3                |
| Comamonas sp.                  | 1                     | 1                 | 5*               |
| Comamonas testosteroni         | 1                     | 2                 | 2                |
| Cupriavidus metallidurans      | 0                     | 0                 | 1                |
| Elizabethkingia meningosepticum| 1*                    | 1                 | 0                |
| Pseudomonas sp.                | 1                     | 1                 | 0                |
| Pseudomonas aeruginosa         | 0                     | 0                 | 1*               |
| Pseudomonas putida             | 1*                    | 2*                | 1*               |
| Pseudomonas nitroreducens      | 0                     | 1                 | 0                |
| Pseudomonas plecoglossicida    | 0                     | 1*                | 0                |
| Pseudomonas staminea           | 0                     | 1                 | 0                |
| Pseudomonas talassii           | 0                     | 0                 | 5*               |
| Pseudomonas veronii            | 6*                    | 0                 | 0                |
| Sphingomonas sp.               | 0                     | 0                 | 1                |
| Stenotrophomonas maltophilia   | 0                     | 1                 | 0                |
| Stenotrophomonas sp.           | 0                     | 0                 | 1*               |

Species that showed anti-*Bd* activity are marked with an asterisk. Each cell indicates the number of individuals carrying a bacterial species. N = 8, 5, 7 toads respectively. doi:10.1371/journal.pone.0044832.t001

Figure 2. Differences in *Bd*-growth averaged from all anti-*Bd* bacterial isolates on each frog species (color code). Growth is estimated at three distances (quartiles 25, 50 and 75) from the bacteria by using spline regression models. Left: original variation as summarized by boxplots and average lines. Right: means for each frog-quartile combination as estimated from the corresponding Manova model. *Atelopus elegans* is the only species we found infected with *Bd* in its natural habitat. doi:10.1371/journal.pone.0044832.g002
Also, strain-level differences in bacteria and overall community structure may prohibit probiotics discovered on one frog species from persisting on another one. For example, *Janthinobacterium lividum*, a bacterium isolated from North American amphibians, failed to prevent or delay mortality in *Bd* exposed individuals of *Atelopus zeteki* [36]. Likewise, we cannot rule out a synergistic or detrimental anti-*Bd* effect of the whole consortium of skin microbiota, because we only evaluated the effects of individual bacteria against *Bd*. It is well known that bacterial consortia are frequently established to carry out several processes that are otherwise harder to accomplish when grown alone [52], for example the oxidation of anaerobic methane [53,54], the metabolism of explosive compounds [55] and the enhancement of bioremediation strategies [56], among others. It has been shown that disruption in antifungal microbial communities is likely to lead to a breakdown of the protective effects of beneficial microorganisms and may lead to disease emergence [57]. Further studies are thus needed to better understand how the bacterial symbionts of frogs’ skin interact among themselves and with their amphibian hosts, and how the activities performed by the skin bacteria could benefit diseased frogs.

Colombia has one of the most diverse amphibian faunas of the world with 751 described species [58], but the current conservation status of many populations and species remains unknown. This study is at the forefront of attempts to seek local bacteria involved in resistance to amphibian chytridiomycosis [59]. We found that amphibian skin microbiota is an important component of disease resistance, and moreover, we describe Neotropical bacteria that provide promising avenues for disease mitigation. In particular, probiotic therapy may be applied as a management tool to reduce the vulnerability of Neotropical amphibians to the devastating effects of *Bd* as has been demonstrated with frogs from temperate zones, where the treatment with beneficial bacteria has been shown to be highly successful to protect susceptible amphibian species from *Bd* infections [59].

Acknowledgments
For assistance in the field we thank A. Batista, G. Corredor, A. J. Crawford, J. A. Hernández, J. Méndez and C. Silva. We thank A. Blasco, J. C. Cubillos, A. Gómez, H. M. Guevara, A. Muñoz, V. Ramírez and M. Villate for assistance in the laboratory. We would like to thank A. J. Crawford, D. C. Woodhams and two anonymous reviewers for helpful comments and suggestions that greatly improved this manuscript.

Author Contributions
Conceived and designed the experiments: SVF CS AA. Performed the experiments: SVF CS MEC EMM. Analyzed the data: SVF CS AA. Contributed reagents/materials/analysis tools: SVF SR AA. Wrote the paper: SVF CS AA. Collected the data: SVF CS.

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