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Programa de Pós-Graduação em Biotecnologia
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Tese

Prospecção funcional e potencial biotecnológico de bactérias associadas a pele de anuros do Quadrilátero Ferrífero

Isabella Ferreira Cordeiro

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia, Núcleo de Pesquisa em Ciências Biológicas da Universidade Federal de Ouro Preto, como requisito à obtenção do título de Doutora em Biotecnologia. Área de concentração: Genômica e Proteômica.

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RESUMO

A contaminação por metais e metaloides tóxicos, natural ou mediada por ação antrópica, tem se tornado um grave problema ambiental, acarretando danos a diversos seres vivos, em especial ao homem. Porém, alguns organismos presentes em distintos níveis tróficos são mais sensíveis a estes contaminantes, e um bom exemplo disso são os anfíbios. Por apresentarem tecido epitelial altamente permeável sofrem mais pela ação destes compostos, o que os colocam, na maioria das vezes, como potenciais bioindicadores de contaminantes. O presente trabalho teve como objetivos prospectar bactérias da pele de anfíbios anuros capazes de tolerar ao arsênio, bem como investigar possíveis relações desta tolerância como mecanismos práticos de aplicação biotecnológica. Para isso, duas investigações paralelas foram conduzidas. Num primeiro momento, isolados bacterianos advindos exclusivamente de anuros de um ambiente contaminado por metais pesados (Estação Ecológica do Tripuí - Ouro Preto, MG) foram avaliados. Para esta abordagem ficou constatado que espécies que possuiam íntimas associações com a água durante seu ciclo reprodutivo apresentam microbiota cultivável mais tolerante e ao mesmo tempo com maior potencial de produção de biofilme, contrapondo-se a microbiota advinda de animais que apresentam menor contato com a água. Considerando que os ambientes aquáticos em áreas contaminadas possuem maior disponibilidade para contato com os organismos vivos, tais resultados são indicativos que tal tolerância e adaptação fisiológica podem contribuir fortemente para a manutenção da sobrevivência da espécie anura quando em contato com estes ambientes no Quadrilátero Ferrífero (QF). Em um segundo momento, a microbiota cultivável associada a cinco espécies da região do QF foi comparada com a microbiota cultivável associada às mesmas espécies, porém advindas de um ambiente não contaminado, localizado a 320 km de distância do primeiro, no Município de João Neiva, ES. Os resultados evidenciaram que a microbiota proveniente das espécies do QF apresentam uma maior tolerância ao arsênio e foram capazes de proteger os anuros da permeabilidade deste metalóide, o que nao foi observado na microbiota advinda das espécies de ambiente não contaminado. Tais dados são indicativos da contribuição desta microbota na manutenção da sobrevivência dos anuros, reiterando os dados apresentados pela primeira abordagem. Como conclusão, os resultados auxiliaram a melhor compreensão desse modelo de interação, bem como classificam potenciais isolados com aplicações biotecnológicas que podem ser melhor exploradas quanto aos possíveis mecanismos moleculares associados a esta notória tolerância.

ABSTRACT

Contamination by toxic metals and metalloids, natural or mediated by human action, has become a serious environmental problem, causing damage to various living beings, especially man. However, some organisms present at different trophic levels are more sensitive to these contaminants, and amphibians are a good example of this. As they present highly permeable epithelial tissue, they are more susceptible to the action of these compounds, which places them, in most cases, as potential bioindicators of contaminants. The present work aimed to: prospect bacteria from the skin of anuran amphibians that are able to tolerate arsenic as well as investigate possible mechanisms associated with this tolerance as practical mechanisms of biotechnological application. For this, two parallel investigations were established. At first, bacterial isolates coming exclusively from anurans from an environment contaminated by heavy metals (Tripuí Ecological Station - Ouro Preto, MG) were evaluated. For this approach, it was found that species that have a close association with water during their reproductive cycle have more tolerant cultivable microbiota and at the same time with greater potential for biofilm production, in contrast to the microbiota from animals that have less contact with water. . Considering that aquatic environments in contaminated areas have greater availability for contact with living organisms, these results are indicative that such tolerance and physiological adaptation can strongly contribute to the maintenance of the survival of the anuran species when in contact with these environments in the QF. In a second moment, the cultivable microbiota associated with five species from the QF region was compared with the cultivable microbiota associated with the same species, but from an uncontaminated environment, located 320 km away from the first, in the Municipality of João Neiva, ES The results showed that the microbiota from the QF species present a greater tolerance to arsenic and are able to protect the anurans from the permeability of this metalloid, not observed for microbiota from the species from an uncontaminated environment. Such results are indicative of the contribution of this microbot in the maintenance of anurans survival, reiterating the results presented by the first approach. In conclusion, the results helped to better understand this interaction model, as well as classify isolated potentials with biotechnological applications that can be better explored.

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O verdadeiro homem mede a sua força quando se defronta com o obstáculo.
Antoine de Saint-Exupéry

Sumário

1-Introdução

1.1-Quadrilátero Ferrífero

Localizado no Centro-Sul de Minas Gerais, o Quadrilátero Ferrífero (QF) é uma região que apresenta um bloco de estruturas geológicas do Pré-Cambriano, cuja forma se aproxima a um quadrado e atinge uma área de aproximadamente 7200 km^2 (Crespo, 2016). Tais estruturas são elevadas em seus quatro lados por erosão distinta, o que leva a altitudes de 1300 a 1600 metros (Barbosa et al., 1967). Variações de altitude acarretam o desenvolvimento de microclimas com temperatura e umidade bem diferentes da temperatura média anual da região, sendo o clima temperado com duas estações bem definidas: inverno seco e verão chuvoso (Silva, 2007). Além disso, a alta geodiversidade existente na região contribue para a existência de diversos tipos de paisagens no QF, variando de floresta decídua semi-tropical a campos rupestres, os quais se destacam por representar um mosaico vegetacional que determina uma ampla variedade de micro-habitats, contendo solos superficiais e ácidos (pH ~ 4,0), com níveis tóxicos de metalóides e metais pesados (Jacobi e Carmo, 2012).

Diante destas características únicas, o desenvolvimento do ecossistema do QF originou comunidades extremamente adaptadas (Jacobi et al., 2007), sendo definido como área de importância biológica especial pela presença de ambientes considerados como potencial hotspot de biodiversidade. Uma vez que, a região do QF é em sua maior parte demarcada como área prioritária para conservação da diversidade da Herpetofauna de Minas Gerais (Drummond et al., 2005), e apresenta relevante endemismo de anuros (Crus e Feio, 2007) (Figura 1), destaca-se a importância da escolha desses anfibios como objeto de estudo desse trabalho.

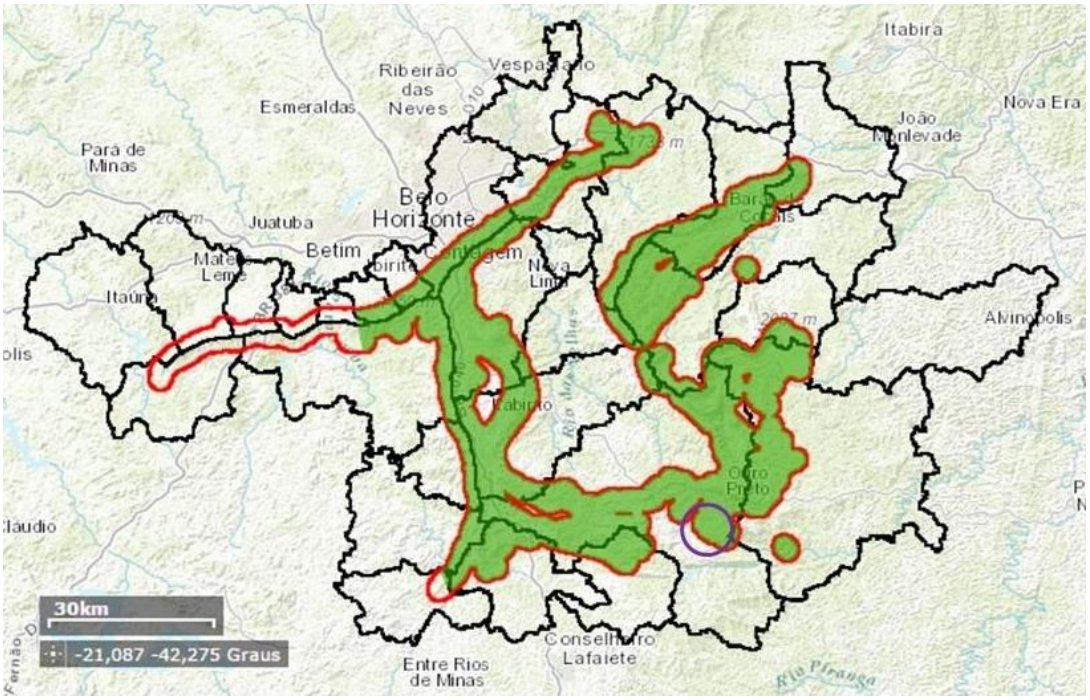

Figura 1: Área prioritária para conservação da diversidade da Herpetofauna de Minas Gerais na região do QF. Em preto destacam-se os limites municipais onde o QF está inserido, representado pelo contorno vermelho. Em verde destaca-se toda a região que se apresenta como área prioritária de conservação da herpetofauna mineira. O círculo roxo destaca a inserção da Estação Ecológica do Tripui (EET) neste contexto (Figura adaptada do atlas do Instituto Prístino).

1.2- Os anuros

Os anfíbios pertencem a classe Amphibia, a qual apresenta as ordens Gymnophiona (cecílias), Caudata (salamandras) e Anura (sapos, rãs e pererecas), sendo essa última ordem alvo do presente estudo (Colombo e Zank, 2008). Os anuros são considerados um grupo altamente sensível a qualidade do meio ambiente, como a qualidade da água (Toledo, 2009). Tal sensibilidade está relacionada às características da pele que é fina, permeável e altamente vascularizada, que além de protegês-los de intervenções abióticas e bióticas, atua como órgão com função respiratória (Toledo, 2009).

A pele dos anuros é adaptada a exercer diversas funções desde proteção, respiração, osmorregulação e termorregulação (Nascimento et al., 2003). Ao desempenhar a função de proteção, a pele atua como defesa primária tendo como principais barreiras, as glândulas dérmicas que secretam moléculas bioativas e a comunidade microbiana (Zasloff, 2002). A presença de comunidades microbianas sobre

a pele dos anfíbios já foi constatada em estudos anteriores (Harris et al., 2006). No entanto, o papel do ambiente na colonização e a composição da microbiota cutânea, bem como os possíveis potenciais biotecnológicos associados a esses microrganismos têm sido pouco investigados. E tal cenário foi motivador para o desenvolvimento desta tese.

1.3- O papel do ambiente na colonização e composição da microbiota cutânea

A comunidade microbiana é caracterizada por um conjunto de microrganismos que habita determinado hospedeiro (Gause, 2021). Tais microrganismos desempenham um papel fundamental na construção da biosfera e estão continuamente sujeitos a variações das condições ambientais (Gause, 2021). Nesse cenário, estudos recentes têm descrito a importância da interação existente entre a microbiota e o hospedeiro. Segundo Assis (2012), uma importante interação microbiota-hospedeiro é a comunidade de microrganimos presente no tecido cutâneo de anfibios anuros, e o habitat é um componente considerável na colonização dessa microbiota. Desta forma, alterações na temperatura, tipo de radiação solar, pH, umidade, e, até mesmo, variáveis do solo e da água podem contribuir para composição da microbiota (Assis, 2012).

Dentre as variáveis químicas e físicas, as quais os microrganismos são suscetíveis, se destacam os xenobióticos e metais pesados (Giller et al., 1998). Esses compostos são capazes de desencadear uma sequência de mecanismos moleculares que induzem danos celulares que culminam, quando de maneira cumulativa, na morte da comunidade microbiana e consequentemente na morte do anfíbio (Hacioglu e Tosunoglu, 2014). No entanto, os anfíbios, em relação a exposição de variantes ambientais, possuem uma particularidade, pois apresentam em seu ciclo de vida uma fase aquática e outra terrestre, além de tecido epitelial altamente permeável (Feder e Burggren, 1992).

Em relação aos compostos danosos aos organismos vivos, estudos envolvendo mercúrio e arsênio têm se destacado (Bertin et al., 2011). Por ser um metaloide de ampla distribuição, pode ser encontrado no solo e água, e é liberado para o ambiente naturalmente ou por meio da erosão ou intemperismo de solos e rochas (Wang e Zhao, 2009). Entretanto, os fatores que mais elevam a disposição do referido metaloide nos ambientes são promovidos pelas atividades antropogênicas (Dembitsky e Rekanza, 2003). No Quadrilátero Ferrífero (QF), região caracterizada como um dos maiores polos mundiais de obtenção de minérios, e referência para este estudo, o arsênio encontra-se em

associação com as rochas auríferas sulfetadas, principalmente a arsenopirita (FeAsS) (Borba, 2002).

Em estudo anterior desenvolvido na região do QF por nossa equipe (Cordeiro et al., 2019), constatou-se que a tolerância de isolados bacterianos prospectados da pele de anuros da Estação Ecológica do Tripuí, área naturalmente contaminada por arsênio. Os resultados auxiliaram a melhor comprensão do modelo de interação microbiota-hospedeiro, tal como indicaram isolados bacterianos com tolerância ao arsênio e capacidade de produção de biofilme que merecem ser investigados, uma vez que a tolerância de microrganismos a contaminação, pode ser associada a mecanismos de resistência (Hacioglu e Tosunoglu, 2014).

1.4- Isolados bacterianos tolerantes a moléculas do ambiente

Populações de microrganismos podem desenvolver resistência aos fatores ambientais, o que os capacita para viver expostos sob determinadas concentrações destas moléculas, a exemplo dos metaloides e metais pesados (Salle, 1961). Tal característica é primordial nos processos adaptativos durante a ocupação de microambientes adversos (Salle, 1961).

Respostas fisiológicas de isolados bacterianos obtidos de anfíbios expostos à metais pesados já foram objeto de estudo, em especial, Hacioglu e Tosunoglu (2014) relatam a tolerância destes isolados a quatro metais pesados (zinco, cobre, cromo e manganês) obtidos de espécies de anfíbios, correlacionando a tolerância com mecanismos de resistência.

A capacidade de tolerância associada a mecanismos de detoxificação faz com que esses microrganismos possam ser utilizados em processos de biorremediação que transformam ou removem resíduos tóxicos ambientais (Abdelatey, 2011). Sendo portanto, considerados importantes recursos biológicos de aplicação em diversificados seguimentos industriais.

Em estudo anterior (Cordeiro et al., 2019) realizado no Quadrilátero Ferrífero, região de formação geológica rica em depósitos arseníferos, onde a intensa atividade de mineração aumenta o risco de contaminação por elementos tóxicos (Nascimento, 2018), foi demonstrada a resistência ao arsênio de isolados bacterianos prospectados da microbiota de anuros. Visto que a resistência ao metaloide pode estar associada a

mecanismos de detoxificação (Hall, 2002), o objetivo do presente estudo busca dar continuidade a essa investigação com o intuito de avaliar a influência do ambiente na composição da microbiota, bem como os possíveis potenciais biotecnológicos associados a essas bactérias.

2- Objetivos

Objetivo geral:

Investigar a tolerância ao arsênio de isolados bacterianos prospectados da pele de anfíbios anuros do Quadrilátero Ferrífero, bem como avaliar o potencial biotecnológico desses isolados.

Objetivos específicos:

- Isolar o maior número possível de bactérias cultiváveis associados ao tecido epitelial de diferentes espécies de anuros do QF, para formar um banco de bactérias.
- Dar continuidade a investigação do papel do ambiente na tolerância ao arsênio de isolados bacterianos advindos da microbitota cutânea de anfíbios anuros
- Avaliar a tolerância desta microbiota cultivável com foco no desenvolvimento de aplicações biotecnológicos.

3- Estrutura da tese

Para facilitar a descrição dos resultados e seguindo as resoluções de redação da tese do Programa de Pós-Graduação em Biotecnologia da UFOP, as seções metodologias, resultados e discussão foram agrupadas em distintos capítulos. A ordem segue a cronologia dos trabalhos desenvolvidos ao longo do estágio doutoral, e os respectivos produtos deste estudo foram incorporados como parte dos respectivos capítulos.

O capítulo I versará sobre a associação entre o estilo de vida de anuros e a tolerância de bactérias associadas ao tecido cutâneo de anfíbios anuros em ambiente contaminado por arsênio (proposta de continuidade de trabalhos preliminares defendidos ao longo do mestrado).

O capítulo II versará sobre o papel do ambiente na tolerância de bactérias associadas ao tecido cutâneo de anfíbios anuros em ambiente contaminado por arsênio em comparação à tolerância de microbiota cultivável advindo de animais coletados em ambientes não contaminados por este metalóide.

E o capítulo III versará sobre o potencial biotecnológico de bactérias associadas ao tecido cutâneo de anuros da Estação Ecológica do Tripuí, Quadrilátero Ferrífero, MG (uma análise preliminar).

3.1- Capítulo I: Estilo de vida de anuros e a tolerância de bactérias associadas ao tecido cutâneo em ambiente contaminado por arsênio

Os dados contidos neste capítulo estão apresentados na íntegra em um artigo científico publicado na revista *Herpetology Notes* em 30 de outubro de 2019.

Título: A resistência ao arsênio da microbiota cutânea está associada ao estilo de vida dos anuros no Quadrilátero Ferrífero, Minas Gerais, Brasil

Isabella Ferreira Cordeiro, Natasha Peixoto Fonseca, Érica Barbosa Felestrino, Washington Luiz Caneschi, Maria Rita Silvério Pires e Leandro Marcio Moreira.

Este artigo é resultado advindo do objetivo de prospectar a microbiota associada ao tecido cutâneo de anfíbios anuros e analisar a influência da presença do arsênio no ambiente na tolerãncia desses isolados bacterianos. Parte dos dados deste artigo foi obtida durante meu mestrado, cuja conclusão se deu após dois anos de ingresso no doutorado.

Em resumo, a composição da microbiota cutânea de anfíbios anuros sofre influência dos microhabitats que esses animais ocupam. No presente estudo, foi investigada a tolerância de isolados bacterianos prospectados da pele de anuros da Estação Ecológica do Tripuí, uma área do Quadrilátero Ferrífero, Minas Gerais, naturalmente contaminada por arsênio. Um total de 328 isolados bacterianos foram obtidos de 21 indivíduos pertencentes a sete espécies de anuros capturados em diferentes microambientes e divididos em dois grupos: maior contato com a água e menor contato com água durante o período reprodutivo. Os resultados indicam que maior número de isolados bacterianos, bem como maior resistência ao arsênio e maior capacidade de produção de biofilme, foram associados a três espécies de anuros que apresentavam maior contato com o arsênio presente na água durante o período reprodutivo. Os resultados auxiliaram a melhor compreensão desse modelo de interação, bem como classificam potenciais isolados com capacidades biotecnológicas.

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Arsenic resistance in cultured cutaneous microbiota is associated with anuran lifestyles in the Iron Quadrangle, Minas Gerais State, Brazil

Isabella Ferreira Cordeiro[1], Natasha Peixoto Fonseca[1], Érica Barbosa Felestrino[1], Washington Luiz Caneschi[1], Maria Rita Silvério Pires[2], and Leandro Márcio Moreira[1,*]

Abstract. The microbiota of the anuran skin contributes significantly to the maintenance and survival of these animals. In the present study, we investigated the composition of culturable microbiota on the skin of frogs from Tripui Ecological Station (TES), a locality in the Brazilian Iron Quadrangle that is naturally contaminated with arsenic. A total of 328 culturable bacterial isolates were obtained from 21 individuals belonging to seven anuran species, captured in different microenvironments within TES. The results indicate that higher numbers of culturable bacterial isolates, as well as higher resistance to arsenic and a higher capacity to produce biofilms, were associated with three frog species that had come into contact with arsenic-contaminated water during their reproductive period. These results raise the possibility that the adaptation of these anurans species to arsenic-laden environments may have a direct correlation with a specialized microbiota.

Keywords. Adaptation; Arsenic resistance; Biofilm; Culturable bacterial prospection; Iron Quadrangle frogs; Skin adhesion

Introduction

Frogs are susceptible to a variety of chemical and physical attributes of their environment, including temperature, pH, water availability, solar radiation, nutrients, and contaminants (Fasola et al., 2015). Due to this susceptibility, one of the most recognized importance of frogs is their potential role as bioindicators of environmental conditions (Beebee and Griffiths, 2005). Human activities, with their concomitant habitat modifications, have contributed to a massive decline in global frog populations and, at the moment, amphibians are the vertebrate class most threatened by extinction (Stuart et al., 2005). The absence of frogs could interrupt the functioning of food webs, leading to ecological imbalance and eventually the loss of other species – including a threat to our own: without frogs, the number of disease-carrying arthropods increases, leading to a significant impact on both natural ecosystems and human-shaped environments (Stuart et al., 2005).

A primary factor in these population declines is man-made pollution, especially as related to the presence of pesticides and heavy metals (Egea-Serrano et al., 2012). Heavy metals are one of the most harmful contaminants, being directly linked to the permeability of the skin in frogs (Egea-Serrano et al., 2012; Hacioglu and Tosunoglu, 2014). Although all heavy metals and metalloids are detrimental to living organisms, studies involving mercury and arsenic have been notable (Lomax et al., 2012; Moriarty et al., 2013).

Arsenic, in particular, is a metalloid with a wide geographical distribution, and it is found in both soil and water (Wang and Zhao, 2009). Two sources cause arsenic contamination, anthropogenic or natural. In Brazil, studies on arsenic contamination are restricted to three main regions associated with mining activity (Mukherjee et al., 2006), which are also described as natural and anthropogenic contaminant sources: (1) the Iron Quadrangle (IQ) in Minas Gerais State; (2) the Ribeira Valley, between Paraná and São Paulo;

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and (3) the region of Santana-Amapá (Borba et al., 2000). In the IQ, a region of great mining activity in Brazil, arsenic is found in association with sulfide-rich auriferous rocks, mainly arsenopyrite (FeAsS) (De Figueiredo et al., 2007). Different studies have verified high concentrations of arsenic in geochemical analyses of water, soil, and sediments of the region and it has been determined that the metalloid is more bioavailable in water (Carmo and Kamino, 2015).

The IQ is defined as an area of particular biological importance because it comprises unique environments, such as ferruginous rupestrian fields, which are considered potential hotspots of biodiversity due to the high endemism of plants and animals (Carmo and Kamino, 2015) (Fig. 1). Recent studies carriedout by our team have demonstrated that the set of cultivable bacteria associated with IQ plants has unique characteristics and biochemical potential that may lie at the root of the specific adaptations of these plants to the various abiotic and biotic factors of the region (Felestrino et al., 2017; Caneschi et al., 2018). Despite this progress among plants, no studies have so far characterised and defined the adaptive potential of bacteria associated with IQ animals. Considering that the microenvironments occupied by frogs differ in environmental characteristics (Haddad et al., 2013), we concentrated our research on the arsenic resistance of the cutaneous microbiota in different microenvironments. Specifically, we studied the possible influence of the presence of arsenic on the microflora of the skin of frogs, comparing species more and less exposed to the metalloid. As environmental conditions exert some influence on the frog skin microbiota (Pollock et al., 2017), a symbiont community that offers protection to such characteristics should be investigated. In this way, we explore and describe the possible role of the cutaneous microbiota as a barrier

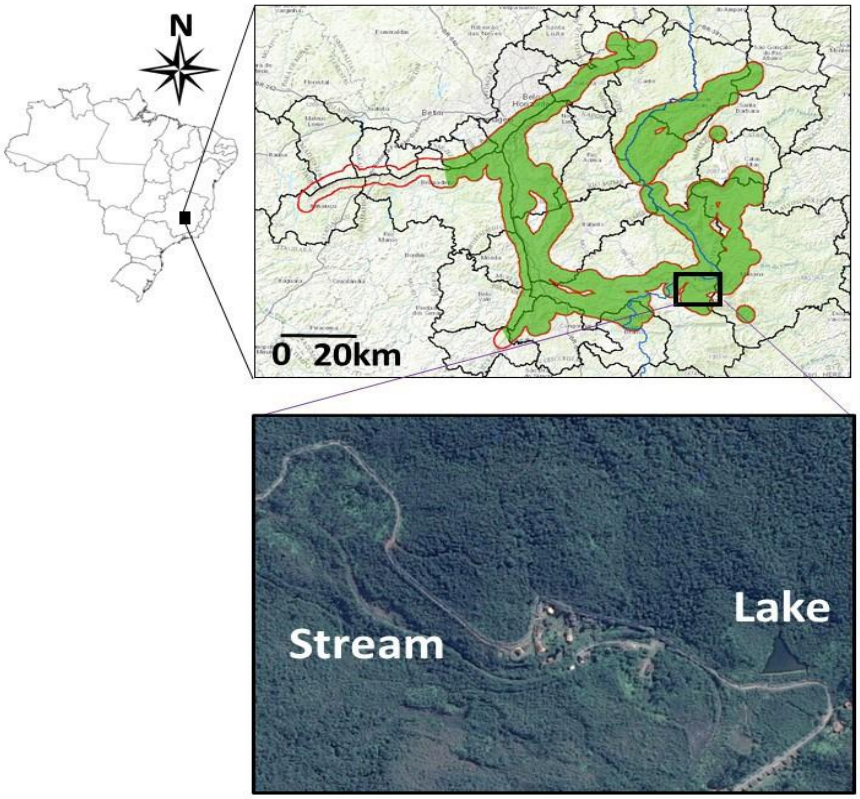

Figure 1. (Top) Location of the Iron Quadrangle (red outline) in Minas Gerais State, Brazil, showing priority areas for the preservation of herpetofauna (green). The approximate location of the Tripui Ecological Station (TES) is indicated by the black rectangle. (Bottom) Satellite image of Tripui Stream and Fortes Lake. Source: Atlas Digital Geoambiental (http://www.institutopristino.org.br/atlas/).

to arsenic contamination by isolating and analysing the largest number of cultivable bacteria associated with the skin of frogs from the Tripuí Ecological Station (TES), a naturally contaminated region within the IQ (Costa et al., 2015).

Materials and Methods

Sampling. Frogs were captured by active nocturnal searches in the TES, located in the IQ region, Ouro Preto Municipality, Minas Gerais (Fig. 1). Sampling points were located around Fortes Lake and along a stretch of Tripuí Stream, within the TES (Fig. 1). Captured individuals were tagged using Visible Implant Fluorescent Elastomer (VIFE) (Grant, 2010).

Anuran species investigated. Three individuals of each species (*Bokermannohyla nanuzae*, *Boana faber*, *Ischnocnema izecksohni*, *Ololygon luizotavioi*, *O. tripui*, *Rhinellacrucifer*, *Vitreoranauranoscopa*) werecollected in February–September 2016 (Table 1) in positions and ecological niches shown diagrammatically in Figure 2. Based on their behaviour and microenvironment at collection, these species were subdivided into two categories. Category 1 includes partially water-dependent or water-independent species (*B. nanuzae*, *I. izecksohni*, *O. tripui*, *V. uranoscopa*) and Category 2 includes species that are water-dependent throughout their reproductive period (*B. faber*, *O. luizotavioi*, *R. crucifer*) (Table 1; Fig. 3).

Boana faber (Wied-Neuwied, 1821).—Males vocalize in marshes or along lakes (Fig. 3.7). Eggs are deposited directly into the water (Table 1) (Martins and Haddad, 1988).

Bokermannohyla nanuzae (Bokermann and Sazima, 1973).—Males vocalize from the trunks and within the foliage of trees and shrubs near flowing waterways (Fig. 3.3). Eggs are deposited directly into the water (Table 1) (Faria et al., 2015).

Ischnocnema izecksohni (Caramaschi and Kisteumacher, 1989).—Males vocalize on the forest floor (Fig. 3.2). The species undergoes direct development, omitting the tadpole stage (Table 1) (Taucce et al., 2012).

Ololygon luizotavioi (Caramaschi and Kisteumacher, 1989).—Males vocalize on stones in small streams or rapids (Fig. 3.6). Eggs are deposited directly into the water (Table 1) (Lourenço et al., 2009).

Ololygon tripui (Lourenço et al., 2010).—Males vocalize from the trunks and from within the foliage of trees and shrubs next to flowing waterways (Fig. 3.4). Eggs are deposited directly into the water (Table 1) (Peixoto et al., 2016).

Rhinella crucifer (Wied-Neuwied, 1821).—Males vocalize in marshes, lakes, or the backwaters of rivers or streams (Fig. 3.5). Eggs are deposited directly into the water (Table 1) (Baldisseri et al, 2004).

Table 1. General features of the species captured in TES to obtain the bacterial isolates, adapted from Haddad et al. (2013). Grey fields are life history stages of the studied frog species during which direct contact with water occurs. The number of bacterial isolates is shown in the NBI column.

Category	Species	Calling sites	Reproductive mode	Tadpoles	NBI
1	*Bokermannohyla nanuzae*	Herbaceous vegetation	Eggs placed directly in water	Exotrophic tadpoles in running water	20
1	*Ischnocnema izecksohnii*	Forest floor	Arboreal eggs	Eggs develop into froglets (no tadpoles)	35
1	*Ololygon tripui*	Herbaceous vegetation	Eggs placed directly in water	Exotrophic tadpoles in running water	18
1	*Vitreorana uranoscopa*	Tree trunks and leaves of arboreal	Arboreal eggs	After hatching, exotrophic tadpoles that drop in running water	22
2	*Boana faber*	Pond	Eggs placed directly in water	Exotrophic tadpoles in constructed basins	60
2	*Ololygon luizotavioi*	Stream	Eggs placed directly in water	Exotrophic tadpoles in running water	74
2	*Rhinella crucifer.*	Pond	Eggs placed directly in water	Exotrophic tadpoles in still water	99

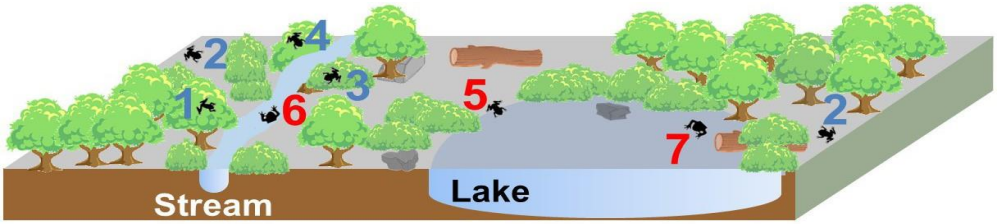

Figure 2. Diagrammatical representation of sampling areas. Numbers are placed to indicate microenvironments where specimens were collected. Species are represented by numbers as follows: (1) *Vitreorana uranoscopa*, (2) *Ischnocnema izecksohni*, (3) *Bokermannohyla nanuzae*, (4) *Ololygon tripui*, (5) *Rhinella crucifer*, (6) *Ololygon luizotavioi*, and (7) *Boana faber*. Numbers are coloured to indicate membership in Category 1 (blue; less contact with water) and Category 2 (red; greater contact with water).

Vitreorana uranoscopa (Müller, 1924).—Males vocalize in trunks and in the foliage of trees and shrubs near streams or rivers (Fig. 3.1). Eggs are deposited on the leaves of branches hanging over water, allowing the tadpoles to fall into flowing water after hatching (Table 1) (Machado et al., 2010).

Collection of microbiota. All frogs were handled manually with sterile gloves and, prior to sample collection, the frogs' bodies were washed one time with sterile water using a wash bottle. Cutaneous microbiotas were obtained from field-caught animals using a sterile swab (K41-0101C, Kasvi Olen, Belo Horizonte, Minas Gerais, Brazil). The swab was rubbed over the entire dorsal and ventral surfaces as well as lateral extensions of each specimen, excluding the head and gular regions, then packed inside a labelled swab-tube.

Isolation and preservation of culturable bacteria. Each swab was inoculated into a 15-ml sterile Falcon tube containing 3 ml of liquid Luria-Bertani (LB) medium (Bertani, 1951) and stirred for 20 s. LB was the preferred medium to favour the growth of the most abundant and faster-growing bacteria present in the frog skin (Medina et al., 2017), whereas M9 or R2A media would merely allow the growth of larger bacterial numbers. We considered faster growth and abundance key factors since a high abundance of metal-resistant isolates may be essential for the survival of the animal in a highly contaminated environment.

Figure 3. Photos of the captured species, including (1) *Vitreorana uranoscopa*, (2) *Ischnocnema izecksohni*, (3) *Bokermannohyla nanuzae*, (4) *Ololygon tripui*, (5) *Rhinella crucifer*, (6) *Ololygon luizotavioi*, and (7) *Boana faber*. The numbers are correlated with collection sites shown in Fig. 2. The black or white scale corresponds 1 cm.

From this culture 1 ml was transferred to a 1.5 ml Eppendorf tube, from which progressive 1:10 dilutions were established until a 10^{10} dilution factor was achieved. Under sterile conditions, 100 µl of these dilutions were inoculated and spread with a Drigalski loop in petri dishes (90 x 15 mm) containing solid LB medium (Fig. 4A). The pH was adjusted to 7.0 along with addition of 70 g/l of thiophanate-methyl (according to the concentration recommended by Viper 700™; Felestrino et al., 2017). Petri dishes were incubated at 28°C for 120 h given that previous studies on microorganisms of the region demonstrated that this temperature provided the greatest diversity and abundance of bacteria (Felestrino et al., 2017; Caneschi et al., 2018). After incubation, bacterial colonies growing on the dishes were isolated.

Bacterial isolation was conducted selecting all colonies that grew on the Petri dish and could be isolated for the dilution factor of 10^{-8}, since this dilution factor resulted in a greater number of colonies without risk of contamination (Fig. 4A). Bacteria were isolated from plates with high dilution factors since in less diluted plates, colony overlap was observed and made differentiation between different bacterial species difficult and increased the risk of contamination (Assis et al., 2017). During isolation, each of the colonies was transferred to a new petri dish that was divided into virtual quadrants using the above conditions. Bacterial colonies were isolated by quadrant and a negative control without inoculation was always included during isolation (Fig. 4B). After isolation, plates were incubated at 28°C for 48 h and then preserved.

For preservation, each bacterial isolate was transferred with a sterile toothpick into a labelled 1.5-ml Eppendorf tube containing 1.0 ml LB broth. Tubes were then incubated at 28°C for 48 h. Glycerol (30% v/v) was added to each tube after growth of bacterial isolates occurred, and samples were stored at –80°C. An additional preservation method consisted of transferring isolates to 96-well round-bottom plates with 100 µl LB broth in each well. Each isolate was duly labelled in one of four 96-well plates. Plates were then incubated at 28°C for 48 h to allow an assessment of isolate growth. Some wells were filled with only liquid LB medium to act as negative controls. After all these steps, the total number of viable and cultivable bacteria was established for each category (Fig. 4C).

Arsenic resistance assay. Petri dishes (150 x 15 mm) containing solid LB medium and different concentrations of sodium arsenite (1, 5, 10, 15, and 20 mM) were used to assess arsenic resistance. Petri dishes containing only solid LB medium were used as negative controls. The bacterial isolates present in the 96-well plates were transferred to petri dishes with a multi-replicator and incubated at 28°C. Pilot tests showed that during 12-day experiments bacteria continued to grow in media containing arsenic. Therefore, photographic records were obtained at 48-h intervals over 12-d periods in order to monitor growth. Resistance was confirmed by the growth of the bacterial colony at a given analysed concentration (Fig. 5).

Biofilm production assay. The biofilm production assay was based on the O'Toole and co-authors protocol (O'Toole et al., 2000), with adaptations described below. The bacterial isolates in the 96-well round-bottom plates containing liquid LB medium were transferred with a 96-point manual microplate replicator and incubated at 28°C for 24 h. After this time, bacterial concentrations were adjusted to an approximate spectrophotometric optical density (OD) at 600 nm wavelength (OD600) of 0.3 and transferred to new 96-well plates containing liquid LB medium, where they were maintained under the same growth conditions as before. After incubation, each plate was washed twice with distilled water. After 1 h of drying at room temperature (21–24°C), 125 µl

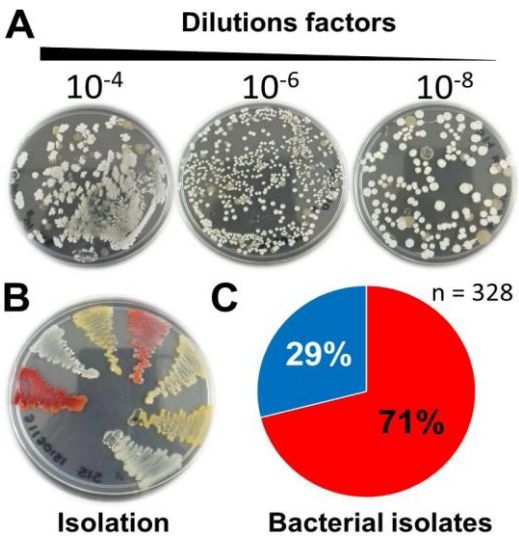

Figure 4. (A) Inoculated petri dishes containing solid LB medium and bacterial streaks at the indicated dilutions. (B) Isolation of culturable bacteria on solid LB medium, showing seven isolates and the control area. (C) Percentage of isolates obtained for Category 1 (blue) and Category 2 (red) species. The difference between these groups is highly significant ($P < 0.001$).

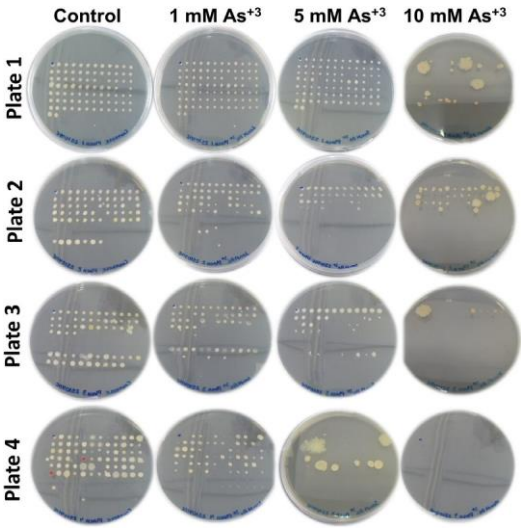

Figure 5. Resistance of the bacterial isolates present in 96-well plates analysed in Petri dishes (150 x 15 mm) containing solid LB medium with different concentrations of sodium arsenite (1, 5, 10, 15 and 20 mM).

of crystal violet solution (10% g/v) was added to each of the 96 wells, and plates were then incubated for 45 min. After 1 h of drying at room temperature (21–24°C), another wash with distilled water was performed, followed by the addition of 125 µl of 95% ethanol and incubation for another 45 min. After this secondary incubation, the well solutions were assessed using a Victor X3 spectrophotometer (Perkin Elmer, Waltham, Massachusetts, USA) at a wavelength of 550 nm to measure absorbance. For this assay, wells containing only liquid LB medium were considered as negative controls, whereas the positive control had known biofilm producers in the respective well. Based on the absorbance values obtained, the bacterial isolates were classified arbitrarily into three categories by calculating the average of two experimental replicates: (1) non-biofilm producers (OD ≤ 0.19); (2) moderate producers (OD between 0.20 and 0.30); and (3) high biofilm producers (OD ≥ 0.31).

Statistical analysis. The data obtained on the number of bacterial isolates of each species per treatment were converted into percentages to improve the comparison between treatments. To test the resistance of bacterial isolates to different concentrations of arsenic and their biofilm production capacity, two generalized linear models (GLMs) were applied (Knutie et al., 2017). The first model used the percentage of resistance of the isolate to arsenic as a dependent variable and the classification of the two frog categories and the interaction between them as an independent variable. The second GLM used the percentage of the isolates involved in biofilm production (non-producers, moderate producers, high producers) as a response variable and the classification of the two frog groups and the interaction between them as an independent variable. All applied models were submitted to residual analysis to evaluate adequacy of the error distribution (Crawley, 2013). In all models, non-significant independent variables ($P < 95\%$) were eliminated from the analyses to obtain an adequate minimum model (Crawley, 2013). All analyses were performed using R software (R Development Core Team, 2013).

Results

Characterization of the microbiota. A total of 328 culturable bacterial isolates were cultured from 21 frogs. For frogs grouped in Category 1, a lower number of bacteria were isolated from the skin, corresponding

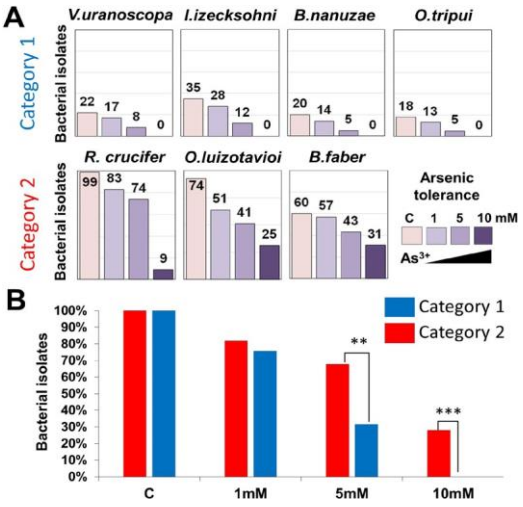

Figure 6. (A) Analysis of arsenic resistance in bacterial isolates obtained from the skin of each studied frog species, where C is the control, and 1, 5 and 10 are the concentrations of arsenic in mM. (B) Bar graph showing the percentage of arsenic-resistant isolates for each group of species. Significance at $P < 0.01$ and $P < 0.001$ are indicated by two (**) and three (***) asterisks, respectively. Colours represent species categories with higher (red) or lower (blue) contact with water, where C is the control, and 1, 5 and 10 are the concentrations of arsenic in mM.

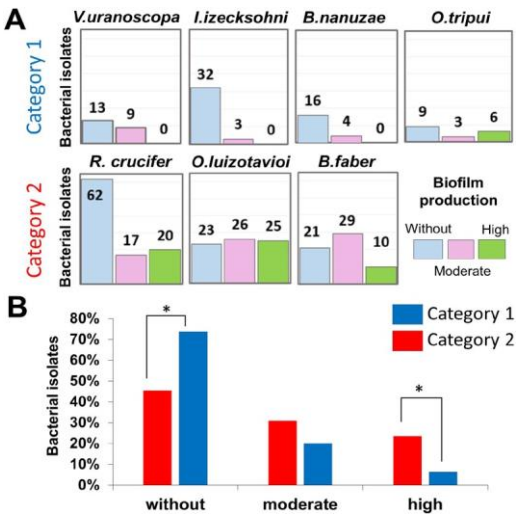

Figure 7. (A) Analysis of biofilm production by bacterial isolates obtained from the skin of each studied frog species. (B) Bar graph showing the percentage of biofilm production by isolates for each category of frog species. The asteristk (*) indicates significance at $P < 0.05$. Colours represent species categories with higher (red) or lower (blue) contact with water.

to 29% (95/328) of the total bacterial isolates (Fig. 4C). Furthermore, the isolates demonstrated lower resistance to arsenic (withstanding only 1 mM and 5 mM concentrations; Fig. 6), and their ability to produce biofilm was reduced (Fig. 7). *Ischnocnema izecksohni*, a species whose reproductive cycle is entirely independent of water, had the lowest percentage of biofilm-producing cutaneous bacteria (8%; Fig. 7A). *Ololygon tripui* was the species that showed the greatest contact with water among the members of Category 1, and its bacterial flora included 33% (6/18) of high biofilm-producing bacteria (Fig. 7).

Culturable bacterial isolates from the skin of anuran species with greater contact with water were obtained in larger numbers, comprising 71% (233/328) of the total isolates (Fig. 4C), and these isolates were more resistant to arsenic (Fig. 6). In particular, the bacterial isolates obtained from *O. luizotavioi* and *B. faber*were resistant to 10 mM arsenic concentrations and corresponded to 34% (25/74) and 52% (31/60) of the total bacterial isolates of each species, respectively (Fig. 6A). In addition, bacterial isolates from these species demonstrated high biofilm production capacity (Fig. 7).

The percentage of culturable bacterial isolates was significantly higher in Category 2 species ($P < 0.001$; Fig. 4C) than in Category 1 species. In addition, the percentage of bacterial growth decreased significantly with the increase in the concentration of arsenic ($P < 0.001$; Fig. 6B). Variations in the percentages of bacterial isolates were observed among different arsenic concentrations depending on the category of frog species, and percentages of bacterial isolates surviving at 5 mM and 10 mM arsenic concentrations were significantly higher in species with greater contact with water (Category 2, $P < 0.01$; Fig. 6B). Biofilm production analysis revealed that the percentage of bacterial isolates varied among the production classifications ($P < 0.001$; Fig. 7). Significant variation was observed among the different bacterial classification groups depending on frog species categories ($P < 0.05$); the percentage of non-biofilm producing bacterial isolates was higher in frogs requiring less contact with water (Category 1), whereas the percentage of biofilm producing bacterial isolates were higher in Category 2 frogs ($P < 0.01$; Fig. 7B).

Discussion

The analysis of the number of isolates revealed that, although in a smaller number of representative species, it was possible to isolate almost three times more bacterial colonies (233, or 71%) associated with the skin of frogs more dependent on water (Category 2) compared with isolation from species with low water contact (Category 1) (Fig. 4C).

Of the 328 cultured bacterial isolates, approximately 80% and 40% presented resistance to arsenic concentrations of 1 mM and 5 mM, respectively (Fig. 6B). Our results suggest that the microbiota was influenced by the presence of arsenic in the environment, since bacteria found in sites with heavy metals tend to develop resistance to contamination by horizontal transfer of genes (Smets and Barkay, 2005).

Environmental changes, including temperature, pH, water availability, solar radiation, nutrients and contaminants are known factors influencing microbial communities (Assis et al., 2017). Thus, behaviour, especially in terms of microhabitats and activity patterns, may also influence the composition of the microbiota within the same environment (Clarke, 1997). Preliminary analyses of the arsenic concentrations in the TES revealed that the concentration in water was four times that found in the soil (Costa et al., 2015). According to our results, the most water-dependent

species (Category 2) were those with the highest percentages of bacterial isolates with high resistance to arsenic. In addition, 100% of the isolates resistant to10 mM concentration were obtained from this category (Fig. 6B).

Despite this, the three species in Category 2 presented distinct skin morphologies. *Ololygon luizotavioi* has skin with variable roughness in the back and belly that is usually quite granular (Lourenço et al., 2009) (Fig. 8). *Boana faber* has smooth skin on the back and granular skin on the belly and, as in *O. luizotavioi*, the mucous glands occur in greater abundance and are responsible for producing a viscous mucus under the skin, keeping them moist (Haddad and Sazima, 1992) (Fig. 8). *Rhinella crucifer* has rough, dry skin on the back and abdomen, and has parotid glands, commonly grouped in the head and neck that contain toxins for protection (Haddad and Sazima, 1992) (Fig. 8). Considering that the three species have different characteristics regarding the skin composition, but similar behaviour during the reproductive period, the greater resistance of the bacteria isolated from these species is a strong indicator of the environmental pressure in the composition of the cutaneous microbiota of these species (Fig. 9).

Although varying widely, the microbiota of frogskin is currently characterized as a component of the protective barrier of the skin (Pessier, 2002; Lips et al., 2006). Therefore, resistance of the microbiota may be associated with the potential detoxification or tolerance to heavy metals (Lapanje et al., 2008). Our data appears to confirm that the microflora on the skin of more water-dependent frog species, which are exposed for a longer duration and to a higher level of contamination, presents a higher resistance to arsenic (Fig 6B; Fig. 9), and we suggest that such resistance help protect the frogs. These results are consistent with the discussion raised in a study by Zhang et al. (2016) whose authors addressed the possibility that microbiota evolves to tolerate heavy metals and that this tolerance may be associated with detoxification mechanisms.

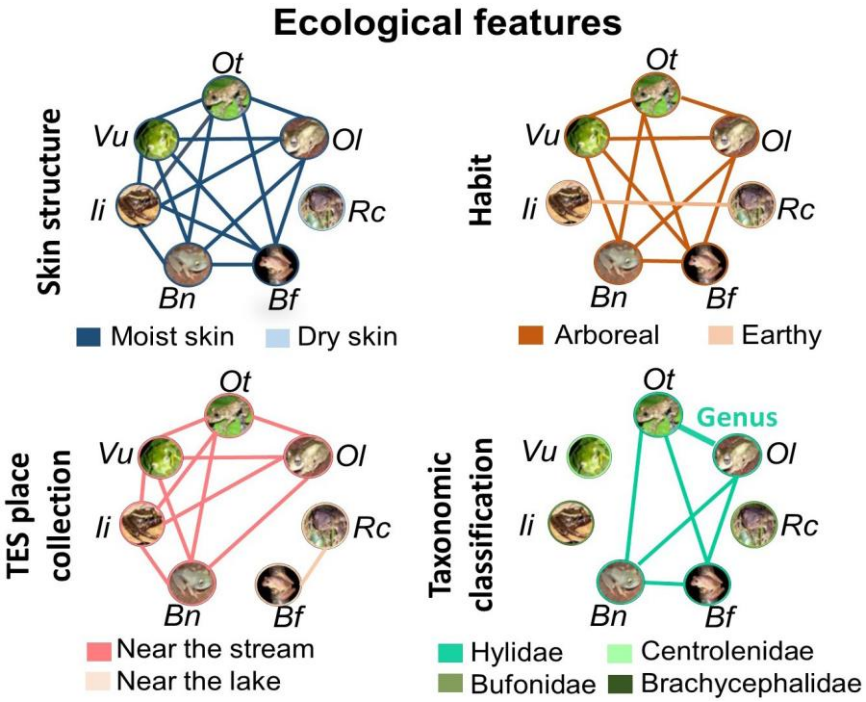

Figure 8. Correlation analysis of ecological parameters for selected frog species in Tripui Ecological Station. Nodes represent the species isolated and investigated, while edges show the relation between them for each of the considered parameters. The thickness of the edges denotes the degree of proximity or relationship between the species for the respective analysed parameters. Abbreviations include Bn (*Bokermannohyla nanuzae*), Bf (*Boana faber*), Ii (*Ischnocnema izecksohni*), Ol (*Ololygon luizotavioi*), Ot (*Ololygon tripui*), Rc (*Rhinella crucifer*), and Vt (*Vitreorana uranoscopa*). Below each investigated parameter is a legend to explain colours and variations observed.

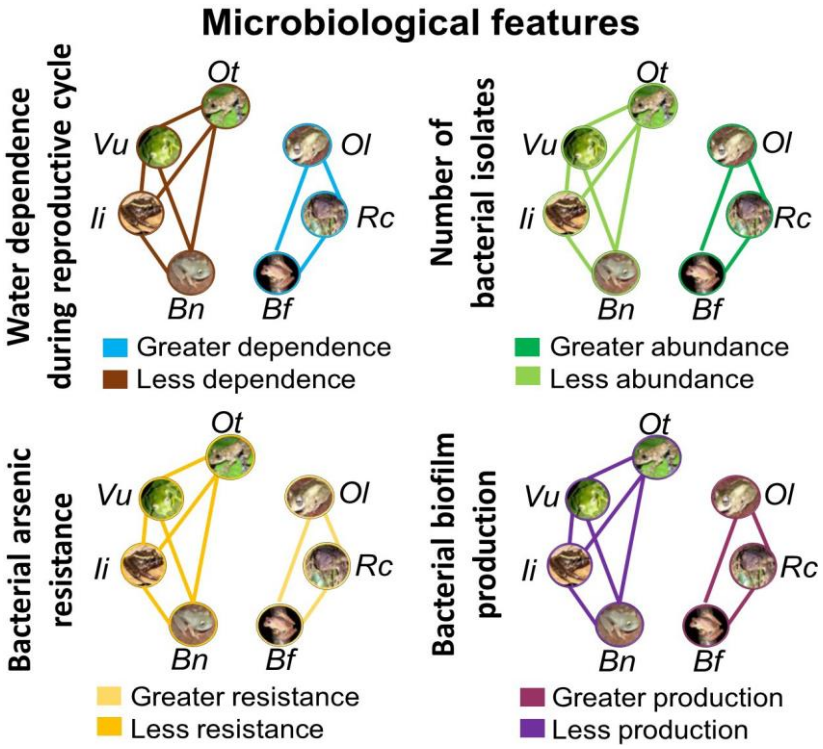

Figure 9. Correlation analysis of microbiological parameters for bacterial isolates obtained from selected frog species in Tripui Ecological Station. Nodes represent the species isolated and investigated, while edges show the relation between them for each of the considered parameters. The thickness of the edges denotes the degree of proximity or relationship between the species for the respective analysed parameters. Abbreviations are as in Fig. 8. Below each investigated parameter is a legend to explain colours and variations observed.

With respect to the adaptive factors inherent to the microbiota, it has been demonstrated that arsenic-resistant microorganisms possess several molecular mechanisms fundamental for exposure to metals, including detoxification of elements or establishment of protective barriers (Achour et al., 2007). Among the mechanisms of detoxification are the sequestrationof extracellular (biosorption) and intracellular (bioaccumulation) metals (Chojnacka, 2010), the production and secretion of chelating molecules such as siderophores (Hider and Kong, 2010), and even biotransformation in less toxic forms of the same metal. The most studied bacterial mechanism as a protective barrier is related to biofilm production (Sarkar and Chakraborty, 2008).

Bacterial biofilms are able to sequester heavy metals from the water (Huang et al., 2000; Labrenz et al., 2000) through an extracellular polymer matrix (EPS), composed of polysaccharides, proteins, and nucleic acids (Kazy et al., 2002). Biofilm assays revealed that bacteria associated with Category 2 frogs had higher biofilm production (Fig. 7B; Fig. 9). The higher biofilm production could act as complementary protection against the use of contaminants through skin, since water acts as an important carrier of these metals. Considering that biofilm production is an adaptive response of microorganisms when exposed to environmental stresses (Singh et al., 2017), different concentrations of arsenic can act as stress inducers and directly affect biofilm production. In addition, another possible explanation for the increase in biofilm production could be the prevention of leaching of bacteria from the frogs.

The data presented in this study demonstrate that the microbial structure of frog skin is influenced by the physicochemical characteristics of the occupied environment, and that this microbiota is clearly a protection mechanism for frogs against the effects of natural contamination. As IQ is one of the regions with

the highest natural contamination by heavy metals in Brazil (Costa et al., 2015), it is possible that the survival and adaptation of frogs in this region have made them highly resistant to metal contamination. The adaptation may in part be due to a specialized microbiota.

Finally, heavy metal-tolerant bacteria almost always exhibit the metabolic and biotechnological potential associated with the detoxification of these compounds, which should be explored both from the point of view of application and the prospect of maintaining the survival of the host species.

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References

Achour, A.R., Bauda, P., Billard, P. (2007): Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. Research in Microbiology **158**: 128–137.

Assis, A.B.D., Barreto, C.C., Navas, C.A. (2017): Skin microbiota in frogs from the Brazilian atlantic forest: Species, forest type, and potential against pathogens. PLoS ONE **12** (7): e0179628.

Baldisseri, F., Caramaschi, U., Haddad, C. (2004): Review of the *Bufo crucifer* species group, with descriptions of two new related species (Amphibia, Anura, Bufonidae). Arquivos do Museu Nacional **62**: 255–282.

Beebee, T.J.C., Griffiths, R.A. (2005): The amphibian decline crisis: a watershed for conservation biology? Biological Conservation **125**: 271–285.

Bertani, G. (1951): Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. Journal of Bacteriology **62**: 293–300.

Borba, R., Ribeiro Figueiredo, B., Rawlins, B., Matschullat, J. (2000): Arsenic in water and sediment in the iron quadrangle, Minas Gerais state, Brasil. Revista Brasileira de Geociências **30**: 554–557.

Caneschi, W.L., Felestrino, E.B., Fonseca, N.P., Villa, M.M., Lemes, C.G.C., Cordeiro, I.F., Assis, R.a.B., Sanchez, A.B., Vieira, I.T., Kamino, L.H.Y., Do Carmo, F.F., Garcia, C.C.M., Moreira, L.M. (2018): Brazilian ironstone plant communities as reservoirs of culturable bacteria with diverse biotechnological potential. Frontiers in Microbiology **9**: 1638.

Carmo, F., Kamino, L. (2015): Ironstones geosystems of Brazil: Priority areas for conservation of geological and biological diversity, cultural heritage and environmental services. Belo Horizonte, Brazil, 3i Editora. [in Portuguese].

Chojnacka, K. (2010): Biosorption and bioaccumulation – the prospects for practical applications. Environment International **36**: 299–307.

Clarke, B.T. (1997): The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biological Reviews of the Cambridge Philosophical Society **72**: 365–379.

Costa, R.D.V.F.D., Leite, M.G.P., Mendonça, F.P.C., Nalini Jr., H.A. (2015): Geochemical mapping of arsenic in surface waters and stream sediments of the quadrilátero ferrífero, Brazil. Rem: Revista Escola de Minas **68**: 43–51.

Crawley, M.J. (2013): The R Book. Second Edition. Chichester, United Kingdom, Wiley.

De Figueiredo, B.R., Borba, R.P., Angelica, R.S. (2007): Arsenic occurrence in Brazil and human exposure. Environmental Geochemistry and Health **29**: 109–118.

Egea-Serrano, A., Relyea, R.A., Tejedo, M., Torralva, M. (2012): Understanding of the impact of chemicals on amphibians: a meta-analytic review. Ecology and Evolution **2**: 1382–1397.

Faria, M., Lourenço, A., Pimenta, B., Nascimento, L. (2015): Morphological variation, advertisement call, and tadpoles of *Bokermannohyla nanuzae* (Bokermann, 1973), and taxonomic status of *B. feioi* (Napoli & Caramaschi, 2004) (Anura, Hylidae, Cophomantini). Zootaxa **3937**: 161–178.

Fasola, E., Ribeiro, R., Lopes, I. (2015): Microevolution due to pollution in amphibians: a review on the genetic erosion hypothesis. Environmental Pollution **204**: 181–190.

Felestrino, E.B., Santiago, I.F., Freitas, L.D., Rosa, L.H., Ribeiro, S.P., Moreira, L.M. (2017): Plant growth promoting bacteria associated with *Langsdorffia hypogaea*-rhizosphere-host biological interface: a neglected model of bacterial prospection. Frontiers in Microbiology **8**: 172.

Grant, E. (2010): Visual implant elastomer mark retention through metamorphosis in amphibian larvae. The Journal of Wildlife Management **72**: 1247–1252.

Hacioglu, N., Tosunoglu, M. (2014): Determination of antimicrobial and heavy metal resistance profiles of some bacteria isolated from aquatic amphibian and reptile species. Environmental Monitoring and Assessment **186**: 407–413.

Haddad, C., Sazima, I. (1992): Anfibios anuros da Serra do Japi. In: História Natural da Serra do Japi: Ecologia e Preservação de uma Área Florestal no Sudeste do Brasil, p. 188–211. Morellato, L.P.C., Ed., Campinas, Brazil, Editora da Unicamp, FAPESP.

Haddad, C.F.B., Toledo, L.F., Prado, C.P.A., Loebmann, D., Gasparini, J.L., Sazima I. (2013): Guia dos Anfíbios da Mata Atlântica – Diversidade e Biologia. São Paulo, Brazil, Anolis Books.

Hider, R.C., Kong, X. (2010): Chemistry and biology of siderophores. Natural Product Reports **27**: 637–657.

Huang, Y.B., Wang, W.H., Peng, A. (2000): Accumulation of Cu (II) and Pb (II) by biofilms grown on particulate in aquatic systems. Journal of Environmental Science and Health, Part A **35**: 575–592.

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3.2- Capítulo II: Papel do ambiente na tolerância de bactérias associadas ao tecido cutâneo de anfíbios anuros em ambientes contaminado e não contaminado por arsênio.

Os dados apresentados neste capítulo estão apresentados na íntegra em um artigo científico em fase de revisão pelos pares na revista PeerJ.

Título: Microbiota cultivável associada à pele contribui para adaptação de anfíbios a regiões naturalmente contaminadas por arsênio

Isabella Ferreira Cordeiro, Camila Gracyelle de Carvalho Lemes, Angélica Sanchez, Ana Karla da Silva, Camila Henriques de Paula, Rosilene Cristina de Matos, Dilson Fagundes Ribeiro, Jéssica Pereira de Matos, Marina Beirão, Leandro Marcio Moreira

Este artigo é resultado da continuação do estudo anteriormente descrito e objetivou investigar o papel do ambiente na composição da microbiota comparando dois ambientes distintos: contaminado e não contaminado por arsênio.

Em resumo, os parâmetros ambientais influenciam os microrganismos presentes no ecossistema, dessa forma, determinados fatores podem ser responsáveis por modular a microbiota cutânea de anfibios anuros. Para esse estudo, cinco espécies de anuros encontradas tanto na região do QF (Minas Gerais), contaminada por metais, quanto na região não contaminada por metais, localizada no município de João Neiva (Espírito Santo), tiveram a microbiota associada aos seus tecidos epiteliais cultivada. A partir do cultivo, os isolados foram investigados quanto à tolerância ao arsênio e sua capacidade de proteger o animal dos danos associados a este metalóide. Os resultados apresentados neste trabalho discutem o uso geral do termo bioindicador, e abrem uma janela de oportunidade para investigar outros processos evolutivos e metabólicos associados a esses animais adaptados a ambientes hostis, bem como possíveis aplicações biotecnológicas que podem advir dessas investigações.

Important declarations

Please remove this info from manuscript text if it is also present there.

Associated Data

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The authors declare that they have no competing interests.

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Culturable microbiota associated with skin contributes to the adaptation of amphibians to regions naturally contaminated by arsenic

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Background. Amphibians are often classified as bioindicators of environmental contamination. However, the presence of amphibians in regions naturally contaminated by metals and metalloids contrasts this perspective, characterizing them as important biological models for studies on adaptation in the presence of this highly harmful contaminant. To understand how the associated microbiota contributes to adaptive processes in amphibians, the arsenic tolerance of the cultivable microbiota associated withanuran skin was investigated. **Methods.** Three specimens of five species from two Brazilian regions were analyzed: one naturally contaminated (Tripuí Ecological Station - TES) and the other without any arsenic contamination (João Neiva-JN). Sterile swabs were rubbed into the skin of amphibians collected in both regions, and cultivable bacteria were isolated. The bacterial isolates were then challenged with increasing concentrations of arsenite. Tolerance data were tabulated, and comparative analyses were established usinga generalized linear model. Then, three metalloid-tolerant and two non-tolerant isolates obtained from species collected in contaminated and non-contaminated areas were investigated for their ability to reduce amphibian exposure to this contaminant. For this, an apparatus was developed from bullfrog skin, which made it possible to investigate whether arsenic from a solution would be able to permeate the skin pretreated with these isolates. **Results.** A total of 646 cultivable bacterial isolates (337 - TES and 309 - JN) were challenged with increasing concentrations of arsenite (As^{3+} 1, 5, 10, 15, and 20mM). Theresults showed that approximately 25% of the microbiota from the species collected in theTES tolerated up to 15 mM of arsenic, with three isolates (0.9%) tolerating up to 20 mM.Most of the microbiota (70.7%) associated with species from JN was sensitive to the lowest

concentration evaluated (1 mM), without any isolate capable of surviving 15 mM of this metalloid. In addition, it was found that such tolerant bacteria can reduce arsenic permeability through the anuran epithelial tissue, thus decreasing the bioavailability of thismetalloid to the vertebrate organism. This effect was not observed in the less tolerant bacteria from the animals collected in uncontaminated regions. **Conclusions.** Culturablebacteria obtained from amphibian epithelial tissue found in areas naturally contaminated by arsenic proved to be much more tolerant to the metalloid and capable of reducing the permeability of this contaminant through the skin of the anuran. Additionally, the results demonstrate that the generalization of amphibians as bioindicators of environmental contamination may be a mistake and that part of the adaptation of these living beings inthe presence of this highly toxic metalloid involves the presence of a specialized epithelial microbiota.

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Abstract

Background. Amphibians are often classified as bioindicators of environmental contamination. However, the presence of amphibians in regions naturally contaminated by metals and metalloids contrasts this perspective, characterizing them as important biological models for studies on adaptation in the presence of this highly harmful contaminant. To understand how the associated microbiota contributes to adaptive processes in amphibians, the arsenic tolerance of the cultivable microbiota associated with anuran skin was investigated.

Methods. Three specimens of five species from two Brazilian regions were analyzed: one naturally contaminated (Tripuí Ecological Station - TES) and the other without any arsenic contamination (João Neiva-JN). Sterile swabs were rubbed into the skin of amphibians collected in both regions, and cultivable bacteria were isolated. The bacterial isolates were then challenged with increasing concentrations of arsenite. Tolerance data were tabulated, and comparative analyses were established using a generalized linear model. Then, three metalloid-tolerant and two non-tolerant isolates obtained from species collected in contaminated and non-contaminated areas were investigated for their ability to reduce amphibian exposure to this contaminant. For this, an apparatus was developed from bullfrog skin, which made it possible to investigate whether arsenic from a solution would be able to permeate the skin pretreated with these isolates.

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Conclusions. Culturable bacteria obtained from amphibian epithelial tissue found in areas naturally contaminated by arsenic proved to be much more tolerant to the metalloid and capable of reducing the permeability of this contaminant through the skin of the anuran. Additionally, the results demonstrate that the generalization of amphibians as bioindicators of environmental contamination may be a mistake and that part of the adaptation of these living beings in the presence of this highly toxic metalloid involves the presence of a specialized epithelial microbiota.

Introduction

Arsenic (As) is one of the most abundant non-essential metalloids found in nature and is considered highly harmful to living organisms (Saha et al. 1999). The damage caused by arsenic is associated with the consumption of contaminated food and water, although in some organisms, contamination can occur due to the permeability of their tissues to this element (Johnson 1971). In recent decades,

human activities have accelerated the exposure of living beings to this contaminant, mediated, for example, by the indiscriminate use of insecticides, herbicides, and preservatives that contain arsenic (Bundschuh et al. 2021). However, in some regions of the planet, arsenic contamination occurs through natural routes (Petrini et al. 2011), arising almost always from the erosive effects of the earth's crust (Borba et al. 2000; Petrini et al. 2011), and consequently, the leaching of these metalloids into watercourses. Arsenic is present in different forms, depending on its oxidation state (-3, 0, +3, and +5), classified as organic or inorganic, and its prevalence depends on pH and redox potential (Eh) (Reid et al. 2020). Inorganic As^{3+} (arsenite) is considered the most toxic form of arsenic because it reacts with the sulfhydryl groups of some proteins and enzymes, preventing their activity (Mohammed Abdul et al. 2015). Among them, we can highlight the antioxidant enzymes of the glutathione class, which protect cells from damage caused by reactive species (RS), and pyruvate dehydrogenase, which converts pyruvate into acetyl CoA (Miller et al. 2002). Although most studies report the damage resulting from exposure to this contaminant, some studies have shown that some species have adaptive tolerance mechanisms when exposed to arsenic. For example, in some plants that survive in these contaminated environments, it is more common to observe such tolerance, reflecting in the discovery of diverse physiological and cellular mechanisms that confer this attribute, which is almost always associated with hyperaccumulation (Briat 2010; Daus et al. 2005; Pence et al. 2000). However, in animals, discoveries regarding arsenic tolerance and adaptation mechanisms still bring many fascinating surprises. Schlebusch et al. demonstrated that a human population inhabiting a naturally arsenic-contaminated region of the Andes in Argentina has an exclusive excretion metabolism of the contaminant, resulting in a probable single nucleotide mutation (SNP) in the AS3MT gene (Schlebusch et al. 2015). The AS3MT gene homolog in prokaryotes (*arsM*) has been reported as an important gene that is horizontally transferred between species, thus granting adaptation to arsenic to the species that receive it. (Chen et al. 2017). Specialized proteins can chelate metals in some fish species, thus reducing tissue damage when exposed to different gradients of these contaminants (Giguère et al. 2006), in addition to activating different detoxification mechanisms (Uren Webster et al. 2013).

In amphibians, the relationship between environmental contamination and tissue damage is even more evident. They are considered bioindicator organisms that are very sensitive to these agents (Adlassnig et al. 2013; Hopkins 2007), as are mollusks and crustacean species that have intimate contact with water (Almeida Rodrigues et al. 2021; Gupta & Singh 2011). Despite receiving such

a classification, questions have arisen over whether or not all species would be sensitive to environmental contamination because the permeability of the epithelial tissue contributes to the bioaccumulation of arsenic and heavy metals. This important theme of adaptive discussion has generated wide debate, and recent studies have shown that some species are highly adapted to high concentrations of metals, whether simulated in the laboratory or under natural conditions (Chen et al. 2009). Such adaptation involves metabolization or excretion of this metalloid under different compositions, especially methylated organoarsenic compounds, mono, and dimethylarsonic acid (Moriarty et al. 2013).

Recently, our team investigated the cultivable microbiota associated with the epithelial tissue of anuran species that have a strict relationship with water, from the region of the Iron Quadrangle (IQ), Brazil (an area naturally contaminated by heavy metals and metalloids). We found that this microbiota is more tolerant to these elements than the microbiota from species that have less contact with water during their reproductive cycle (Cordeiro et al. 2019). Proença et al. corroborated this data in a study involving other species (Proença et al. 2021). In continuation of this preliminary study, the cultivated microbiota associated with the epithelial tissues of five species of anurans from the IQ region, as well as from a non-contaminated area located in the municipality of João Neiva (Brazil), were investigated for arsenic tolerance and their ability to protect the animal from damage associated with this metalloid. The results presented in this work discuss the general use of the term "bioindicator" and open a window of opportunity to investigate other evolutionary and metabolic processes associated with these animals adapted to environments that would be lethal to other species with related characteristics.

Materials & Methods

Selection of areas and anuran species investigated

First, anurans were captured in the region of Estação Ecológica do Tripuí (TES) (an area contaminated by heavy metals), located in the municipality of Ouro Preto in the state of Minas Gerais (Figure 1). Twelve species of isolates were identified: *Bokermannohyla nanuzae, Boana faber, Dendropsophus elegans, Hypsiboas albopunctatus, Hypsiboas polytaenius, Ischnocnema izecksohni, Leptodactylus latrans, Ololygon luizotavioi, Ololygon tripui, Rhinecoorra,* and *Rhinecorcifera*. Second, a capture was carried out in the municipality of João Neiva, and its surroundings, located in the state of Espírito Santo (approximately 370 km from Ouro Preto)

(Figure 1). This region was established as a search site for anura species because of the high preservation of its native forests and the absence of reports of contamination by heavy metals, which allowed the prospecting of isolates of the following 15 species: *Boana faber, Dendropsophus anceps, Dendropsophus elegans, Dendropsophus bipunctatus, Dendropsophus branneri, Dendropsophus minutus, Hypsiboas albopunctatus, Hypsiboas polytaenius, Ischnocnema feioi, Physalemus cuvieri, Rhinella crucifer, Rhinella granulosa, Rhinella schneideri, Scinax fuscovarius,* and *Leptodactylus fuscus*. From the two collections, only these five species were identified in both regions: *Boana faber, Dendropsophus elegans, Hypsiboas albopunctatus, Hypsiboas polytaenius,* and *Rhinella crucifer*. These were selected for the development of this study.

Characterization of sampled regions

The TES is characterized as a transition region between the two Brazilian biomes that are considered global hotspots for biodiversity conservation, the Atlantic Forest, and the Cerrado. Located in the metallurgical zone of the city of Ouro Preto, the state of Minas Gerais,-BR (20°23'45" S e 43°34'33" W) (Myers et al. 2000)**,** in the Tripuí Creek sub-basin, it has an area of 3.37 Km^2 and an altitude that varies between 1280 and 1450 m. Among its geographic characteristics, the climate characterized as Cwb stands out (subtropical highland), with temperate summer and dry winter, annual rainfall close to 1,600 mm, and an average annual temperature of 18ºC (FEMA 1995). In the TES region, the sampling points were located around Lake Fortes along a stretch of Ribeirão Tripuí. This region was chosen as the study area to continue the previous research developed by Cordeiro et al. and to present high levels of heavy metals and metalloids (Borba 2002; Costa et al. 2015; Cruz 2002).

The area not contaminated by arsenic is located around farms in the municipality of João Neiva, state of Espírito Santo, Brazil. The municipality of João Neiva has an area of 272.30 km^2 and is located between latitudes 19°37'20″" and 19°47'57" and longitudes 40°31'37" and 40°20'28"″ W, respectively. In general, its geographic characteristics are a Cwa-type climate (monsoon-influenced humid subtropical), characterized by dry winters and rainy summers. Throughout the year, the temperature generally varies from 17°C to 32°C and is rarely below 14°C or above 35°C. Its territory is largely composed of the Atlantic Forest biome (Luppi et al. 2015; Panceri Fleguer et al. 2007).

Anuran species investigated

Three male individuals of species *Boana faber*, *Dendropsophus elegans*, *Hypsiboas albopunctatus*, *Hypsiboas polytaenius*, and *Rhinella crucifer* (Figure 1) were captured from February to September 2016. The general characteristics of these species are summarized in Table 1. Frogs were captured using an active night search. At both locations, captured individuals were tagged using a visible implant fluorescent elastomer (VIFE) to prevent recapture (Campbell Grant 2008). All procedures were carried out in accordance with licenses granted by ICMBio process n° 29219-4 and IEF license n°UC 087/13.

Microbiota collection

Immediately after capture, the animals were washed with two distilled water baths previously autoclaved in sterilized bottles. Then, the skin microbiota was collected in the field with a sterile swab (Olen Ref K41-0101C). The swab was passed over the skin of the specimen along the entire length of the back, belly, and lateral region, avoiding contact with any other surface not associated with the animal. Disposable and sterile materials were used for this procedure, as described by Cordeiro et al. (Cordeiro et al. 2019).

Isolation, preservation, and cultivation of bacteria

Swabs were taken to the laboratory within 48 h of collection, and each swab was passed on the skin of anurans and placed in a sterile 15 mL Falcon tube containing 3 mL of liquid Luria-Bertani (LB) medium (Bertani 1951). From this first sample, 1 mL was transferred to a 1.5 mL Eppendorf tube. Progressive dilutions at a ratio of 1/10 were established up to the 10^{8th} dilution. Then, 100 µL of the bacterial solution at the 10^{8th} dilution was inoculated and spread with a Drigalski loop in individual Petri dishes containing LB medium, with pH adjusted to 7.0, plus 2 mg/L of Viper 700® (methyl thiophanate), and kept at a temperature of 28°C for 48 h. After this period, all bacterial colonies that grew on these plates were then isolated using sterile wooden sticks in new Petri dishes containing the same medium, incubated for another 48 h at 28°C, and then preserved. For preservation, each isolate was transferred to a previously identified Eppendorf tube containing 1.0 mL of liquid LB medium. The tubes were then incubated for 48 h at 28°C. After this period, glycerol was added to each tube at a final concentration of 30% (v/v). Part of the microbiota used

in this study was reused from the work of Cordeiro and collaborators (Cordeiro et al. (2019), specifically referring to the isolates obtained from the species *Boana faber* and *Rhinella crucifer* collected at TES.

Arsenite resistance assay

To analyze the resistance to arsenic, Petri dishes with solid LB medium at different concentrations of As^{3+} (1 mM, 5 mM, 10 mM, 15 mM, and 20 mM) were used, in addition to a control dish containing only solid LB medium. With the aid of the replicator, the identified isolates were transferred to 96-well plates. They were later replicated in Petri dishes with different concentrations of arsenite and incubated at 28°C using a 96-tip multireplicator. Photographic records were taken at 48h intervals over 12 days to monitor the growth of the isolates. The resistance was assessed by observing colony growth at each concentration.

Skin permeability test

To understand whether the microbiota would reduce the permeability of amphibian skin to arsenic, we developed a rudimentary apparatus that would simulate the environmental conditions and the anuran organism in the presence of contaminants. For this, all materials (test tubes, beakers, rubber bands, a conductivity meter, solutions simulating the animal's bodily fluids, and the environment contaminated by arsenic) were required as summarized and presented sequentially (Figure 2). Five bacteria were selected for these assays, two isolates from JN species tolerant to 1 and 5 mM, and three from TES species tolerant to 5, 10, and 15 mM of arsenite, respectively. Additionally, the *E. coli* strain DH10B was included in the trial as a control. These bacteria were reactivated and applied on solid LB medium in a Petri dish (60 x15 mm) for two days at 28°C. Fragments of bullfrog skin (*Lithobates catesbeianus*) were acquired with the help of a commercial producer responsible for aseptically extracting the skin and keeping them individually packaged and frozen. Once in the laboratory, the skins were thawed at room temperature and cut into square-shaped fragments with 6 cm edges. These skin fragments were then placed in contact with bacterial cultures in Petri dishes, through their external face, and incubated at 28 °C for 24 h. After this period, the skin was removed from the Petri dish and separated. The skin was then tied to the open end of a test tube containing an arsenic solution (5 mM $NaAsO_2$ final concentration), and the external surface of the skin containing the previously adhered bacterial culture was turned towards

contact with the arsenic solution. The tubes were poured into an Amphibian Ringer's solution (ARS - 6.6 g of NaCl, 0.15 g of KCl, 0.15 g of $CaCl_2$, and 0.2 g of $NaHCO_3$ dissolved in 1 L of distilled water (Larsen 2021)), which simulated the animal's fluids. For the test tube to remain stable and maintain the inner surface of the skin in continuous contact with the ARS (suspended 2 cm from the bottom of the beaker), a rigid black paper plate was cut, and the tube was fitted in its center, supporting the weight of the tube by the edges of the beaker, thus limiting the contact of the ARS with the environment. The conductivity meter sensor was inserted in contact with the ARS at established times (0, 1.5, 3, and 24 h), and the conductivity of the solution was measured and tabulated in triplicate.

Indirect evaluation of the bacterial growth profile in the apparatus

In addition to the conductivity, we also evaluated the changes in the turbidity of the arsenic solution and bacterial growth. Turbidity was assessed by observing the change in color of both arsenic and ARS solutions, as is indicative of bacterial growth. These growths were confirmed by the reactivation of bacteria from both solutions in the culture medium. For this, 10 μL of each solution was inoculated and spread with a Drigalski loop in individual Petri dishes containing LB medium, with pH adjusted to 7.0, and kept at a temperature of 28°C for 48 h. Morphology of the colonies was compared with the morphology of the colonies isolated from the respective bacteria investigated.

Statistical analysis

To compare the growth of the skin microbiota between TES and JN amphibians grown on the arsenic medium, we made a generalized linear model (GLM) with the amount of microbiota as the response variable and the microbial growth area , the concentration of arsenic and the interaction between them as the explanatory variables. To test the differences between species, the GLM was the amount of microbiota as a response variable and each species as an explanatory variable. For both models, as the response variable comes from count data, the distribution error used was Poisson (Crawley 2013). All statistical analyses were performed using R (v. 4.0.4) (Team 2021).

Results

A total of 644 bacterial isolates were obtained from the three specimens of each of the five anuran species investigated in both study areas. Of these, 337 were from species from the contaminated

region and 307 from the region from non-contaminated species, with no statistically significant difference (Table 1). However, when the number of isolates for each species in both collection areas was analyzed separately were observed only for the isolates obtained from *Hypsiboas albopunctatus* (with the highest number of isolates in each contaminated area) and *Hypsiboas polytaenius* (with the highest number of isolates in an uncontaminated area) certain difference in sample number (Table 1).

All isolates obtained from the skin of anurans were challenged with increasing concentrations of arsenite in the culture medium to determine the tolerance of the microbiota. The number of isolates tolerant to As^{3+} was higher in the group of species from the contaminated area (TES) at concentrations of 1 and 5 mM As^{3+} (Figure 3A). Only the contaminated area showed isolates tolerant to 10 mM and 20 mM As^{3+}. The microbiotas' tolerance profiles of each species from TES and JN were analyzed individually. It was observed that for all investigated anuran species, the microbiota obtained in the contaminated area was always more tolerant, with microbial growth observed 48 h after exposure to the contaminant (Figure 3B). However, some of these isolates showed positive growth only in the final investigation time (240 and 288 h) in the presence of arsenite. Finally, the largest fraction of bacterial isolates tolerant to 10 and 15 mM of the metalloid came from the species *D. elegans*, *B. faber*, and *H. albopunctatus*, which are species that have greater contact with water during their reproductive cycle (Figure 3B).

For the permeability assays, bullfrog skin was used as a model tissue, in association with five of the 644 bacterial isolates previously investigated, in addition to an *E. coli* strain. Two were selected from the species *H. polytaenius* (JN1) and *B. faber* (JN2), respectively, and were collected from the uncontaminated area. Both were identified as being the most resistant among all isolates obtained from the species in this area. To establish a comparison, two other isolates were incorporated into the assay, coming from the contaminated region (TES1 and TES2). The fifth isolate evaluated was obtained from the species *H. albopunctatus* also collected in the contaminated region, and in this case, also demonstrated one of the highest tolerances observed for arsenic (Figure 4A). It was found that, when incorporated with the external face of the epithelial tissue of bullfrog, the isolate JN1 had greater conductivity in the ARS, as observed for skin associated with *E. coli*, or even skin without any associated bacteria. The change in conductivity for the JN2 and TES1 isolates was slightly smaller but higher than that observed for TES2 and TES3 isolates. For the latter, this change was only observed 3 h

after the start of the test (Figure 4B). Based on this study, we evaluated the rate of bacterial growth in both ARS (simulating fluids of the anuran) and arsenite solution (simulating the environmental conditions). After carrying out the permeability tests, it was found that microbial growth was only observed in samples of the ARS derived from the apparatuses that contained the control skins and treated with isolates from the uncontaminated area. In contrast, skins treated with bacteria from the contaminated area only grew from the system containing arsenite solution (Figure 4B).

Discussion

Amphibians represent a significant group of animals. Despite their notorious importance for ecosystem maintenance (Hocking & Babbitt 2014), amphibian populations in the world show a substantial decline (Alford & Richards 1999). The emergence of diseases, such as chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (Bd) (Scheele et al. 2019)), come as a consequence of the destruction of their natural habitats mediated by anthropogenic action (Alton & Franklin 2017).

Because epithelial tissue is highly permeable to a series of chemical compounds (Llewelyn et al. 2019), amphibians are vulnerable to drought and toxic substances, and are considered critical indicators of ecosystem health (Venturino et al. 2003). However, recent studies and perspectives have shown that this concept needs to be revised to prevent it from being used in a generalized way. In the face of the decline of some amphibian populations, other species notoriously remain or stand out, given their greater adaptive resilience (Pyron 2018). However, we must consider what factors could contribute to this greater resilience. Physiological adaptations have been gaining prominence as some of the most significant factors related to amphibian adaptations in environments with high selective pressure, such as areas naturally contaminated by heavy metals and metalloids. Moryart et al. described, for example, the ability of the American amphibians *Rana clamitans* and Bufo, collected from a site with elevated arsenic concentrations in Nova Scotia, Canada, to transform this metalloid from its highly harmful inorganic state to a less damaging organic state (Moriarty et al. 2013), thereby reducing cellular and metabolic damage.

In addition to physiological adaptations, many studies have focused on understanding the diversity and possible interactions of the microbiota associated with amphibians with adaptive events, thus placing the microbiota study as fundamental to conservation biology studies (Trevelline et al.

2019). Most of this extensive repertoire of works has been using metagenomic approaches, with a focus on the study of dysbiosis induced by the presence of Bd (Flechas et al. 2019; Jani & Briggs 2014; Rebollar et al. 2018; Rebollar et al. 2016; Walke Jenifer et al. 2015). Other studies, however, have revealed that although there is a significant microbiota often found in many species, the levels of bacterial diversity vary greatly across species, and these innate species differences appear to regulate the structure of bacterial communities in amphibians (McKenzie et al. 2012). Therefore, the skin-associated bacteria of amphibians are recognized for their role in defense against pathogens or environmental contaminants, yet their basic ecology is not well understood (Kueneman et al. 2014).

Despite this advance arising from diversity studies based on metagenomics, studies dependent on bacterial culture are still emerging, even when dealing with epithelial tissue, the most widely investigated. Assis and collaborators, for example, investigated 221 bacterial isolates from 188 individuals belonging to 4 species of amphibians and concluded that the environment modifies the microbiota, and that part of these isolates have antimicrobial potential, suggesting a potential aid in protecting the anuran epithelial tissue (Assis et al. 2017). Similarly, a study carried out with anurans from the IQ region (naturally contaminated by metals and metalloids) (Varejão et al. 2011) found that animals with constant contact with watercourses have a more arsenic-tolerant microbiota when compared to animals that only partially depended on water to reproduce (Cordeiro et al. 2019). Additionally, the microbiota associated with animals with constant water contact has a significant capacity to produce biofilms. Biofilm production could aid in the fixation of microorganisms to the animal tissue and impose pronounced protection against contaminants from physicochemical variations in the external environment (Cordeiro et al. 2019). Proença et al. corroborated some of these results in a study of the cultivable microbiota of Perez frogs (*Pelophylax perezi*) collected in regions contaminated and uncontaminated by metals (Proença et al. 2021). This study demonstrated that the cultivable microbiota, although it does not reflect the bacterial diversity of the environment, can contribute to a significant understanding of the ecology and bacterial-animal interaction processes.

To increase our knowledge of the importance of this microbiota and the possible relationship in protecting amphibians from external contaminants in this study, we aimed to investigate the tolerance profile of the cultivable microbiota of the epithelial tissue of amphibian species collected in arsenic-contaminated areas (TES, located on the south face of the IQ, in Minas

Gerais), and non-contaminated areas (João Neiva in Espírito Santo) (Figure 1). We also aimed to assess the ability of this microbiota to protect amphibians from the effects of arsenic. Three individuals were identified and collected from five investigated species, including *Boana faber*, *Dendropsophus elegans*, *Hypsiboas albopunctatus*, *Hypsiboas polytaenius*, and *Rhinella crucifer* (Figure 1 and Table1).

A total of 644 bacterial isolates were obtained and evaluated for arsenic tolerance (Table 1). This metalloid was selected as a reference because it is highly harmful to living organisms (Saha et al. 1999) and is reported to be abundant in the region of the IQ (Borba 2002; Costa et al. 2015; Cruz 2002). Although no significant difference was identified in the sample number of bacterial isolates from the species collected in both regions, it was evident that those from amphibians collected from the contaminated area were much more tolerant to arsenic than those from species collected from an uncontaminated area (Figure 3A). These results suggest that the speciation of the microbiota may have been influenced by the presence of the contaminant, since environmental factors are known to strongly influence the composition and diversity of the microbiota present in the ecosystem or ecological niche (Assis et al. 2017; Yao et al. 2017).

Curiously, the isolates that presented elevated tolerance to the highest concentration of arsenite (10 mM) came from the species *Boana faber*, *Dendropsophus elegans*, and *Hypsiboas albopunctatus*, whose behavior and life cycles are more dependent on water than those of *Hypsiboas polytaenius* and *Rhinella crucifer* (Haddad et al. 2013) (Figure 3B). These data corroborate data previously published by our team (Cordeiro et al. 2019), highlighting that the most water-dependent species were those with the highest percentage of bacterial isolates with high tolerance to metals. Both studies demonstrated that behavior, especially in terms of microhabitats, can also influence the composition of the microbiota, reiterating the role of the microbiota in the adaptation of anurans to contaminated sites (Moriarty et al. 2013).

In an attempt to understand whether greater tolerance could contribute to the supposed protection of the anuran when exposed to environmental contaminants, a rudimentary and low-cost but efficient apparatus was specifically developed for this work (Figure 2). The results showed that the isolates obtained from species collected in an uncontaminated area (JN1 and JN2) and less tolerant to arsenic had a higher rate of arsenic migration from the metalloid solution (simulating the environment) to the ARS (simulating the anurans fluids), obligatorily diffusing through the epithelial tissue. Such diffusion induces a change in conductivity in the

ARS solution because the electrical conductivity is affected by the concentration of ions in the solution (Aziz et al. 2008). It is interesting to note that such data perfectly contrasts with the results obtained from the isolates from species collected in a contaminated area and, therefore, more tolerant to arsenic (Figure 4AB). For isolates TES2 and TES3, the change in conductivity in the ARS solution was much smaller, which corroborates the tolerance profile of these isolates to arsenic and, therefore, the increased protective potential for anurans. A curious fact observed for isolates with greater protective power is that they showed a positive growth rate in the contaminant solution, which could suggest a chemoautotrophic metabolic profile, whereas isolates with lower protection potential presented microbial growth only in ARS (Figure 4C).

These data, in association, even if in a very preliminary way, suggest that such bacteria can present themselves as a primary protective barrier to the epithelial tissue. This allows, among other possible adaptations, amphibians to survive and adapt to these naturally contaminated environments. This hypothesis cannot be extrapolated to amphibian-associated bacteria present in uncontaminated areas, which would likely corroborate the declines in most of these populations when acutely exposed to such environmental contaminants (Figure 5A). Therefore, the presence or absence of resilience of an anuran species to environmental contamination is directly associated with the metabolic and functional capacity of its epithelial microbiota (Christian et al. 2018; Knutie et al. 2017), thus contributing to the success of colonization or decline of a given population (Pyron 2018).

Regarding these results, this work brings up an important discussion about the use of the term “contamination bioindicator” being used in a generalized way to amphibians. Here, we propose that the term should be used sparingly since, as demonstrated in this work, species found in naturally contaminated environments have, in addition to physiological adaptations (Moriarty et al. 2013), microbiota speciation that is essential for their maintenance (Figure 5A).

Finally, although it was not the focus of this work, it is important to highlight that bacteria may have diverse mechanisms for detoxifying metals and metalloids (Figure 5B). Among these, the biofilm production capacity stands out, thus decreasing the diffusion and direct contact of the metal with the animal tissue. Other mechanisms involving the ability to promote redox processes (which could lead to the conversion of a toxic ion into a less toxic state), the possibility of biotransformation (forming organometallic complexes), the efflux of these contaminants out of the

cell, the chelation of these ions by organic molecules, and the increase in the metabolic profile of oxidative stress-associated systems (Outten et al. 2000) are also significant. This context highlights the importance of the microbiota as fundamental to the study and practice of conservation biology (Trevelline et al. 2019) and, in the case of amphibians found in contaminated areas, as excellent models for the study of animal-microbiota interactions, and contaminated areas as hotspots for understanding the evolutionary profiles of associated species.

Conclusions

The tolerance of the microbiota associated with the skin of anurans found in areas naturally contaminated with arsenic seems to play a fundamental role in the adaptation of the host species, contributing to the reduction of tissue permeability to this contaminant. Therefore, the term "bioindicator", generally assigned to amphibians, should be used with moderation. The geographic region and environmental characteristics of these animals should be considered, instead of only the genus or species to which they belong, or the simple fact that they have highly permeable epithelial tissue. This work highlights the anurans that survive in naturally contaminated regions as significant models of study in future investigations into the mechanisms associated with detoxification and metal metabolism, some of which are considered highly harmful to most living beings, as is the case with arsenic.

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References

Adlassnig W, Sassmann S, Grawunder A, Puschenreiter M, Horvath A, and Koller-Peroutka M. 2013. Amphibians in metal-contaminated habitats. *Salamandra* 49:149-158.

Alford RA, and Richards SJ. 1999. Global Amphibian Declines: A Problem in Applied Ecology. *Annual Review of Ecology and Systematics* 30:133-165. 10.1146/annurev.ecolsys.30.1.133

435 Almeida Rodrigues P, Ferrari RG, Kato LS, Hauser-Davis RA, and Conte-Junior CA. 2021. A Systematic
436 Review on Metal Dynamics and Marine Toxicity Risk Assessment Using Crustaceans as Bioindicators.
437 *Biol Trace Elem Res*. 10.1007/s12011-021-02685-3
438 Alton LA, and Franklin CE. 2017. Drivers of amphibian declines: effects of ultraviolet radiation and
439 interactions with other environmental factors. *Climate Change Responses* 4:6. 10.1186/s40665-017-
440 0034-7
441 Assis AB, Barreto CC, and Navas CA. 2017. Skin microbiota in frogs from the Brazilian Atlantic Forest:
442 Species, forest type, and potential against pathogens. *PLoS One* 12:e0179628.
443 10.1371/journal.pone.0179628
444 Aziz Z, van Geen A, Stute M, Versteeg R, Horneman A, Zheng Y, Goodbred S, Steckler M, Weinman B,
445 Gavrieli I, Hoque MA, Shamsudduha M, and Ahmed KM. 2008. Impact of local recharge on arsenic
446 concentrations in shallow aquifers inferred from the electromagnetic conductivity of soils in Araihazar,
447 Bangladesh. *Water Resources Research* 44. https://doi.org/10.1029/2007WR006000
448 Bertani G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. *J*
449 *Bacteriol* 62:293-300. 10.1128/jb.62.3.293-300.1951
450 Borba RP. 2002. Arsênio em ambiente superficial: processos geoquímicos naturais e antropogênico em
451 uma área de mineração aurífera Doutorado em Ciências. Universidade Estadual de Campinas.
452 Borba RP, Figueiredo BR, Rawlins B, and Matschullat J. 2000. Arsenic in water and sediment in the Iron
453 Quadrangle, state of Minas Gerais, Brazil. *Revista Brasileira de Geociências* 30:558-561.
454 Briat JF. 2010. Arsenic tolerance in plants: “Pas de deux” between phytochelatin synthesis and ABCC
455 vacuolar transporters. *Proceedings of the National Academy of Sciences* 107:20853.
456 10.1073/pnas.1016286107
457 Bundschuh J, Schneider J, Alam MA, Niazi NK, Herath I, Parvez F, Tomaszewska B, Guilherme LRG,
458 Maity JP, Lopez DL, Cirelli AF, Perez-Carrera A, Morales-Simfors N, Alarcon-Herrera MT, Baisch P,
459 Mohan D, and Mukherjee A. 2021. Seven potential sources of arsenic pollution in Latin America and
460 their environmental and health impacts. *Sci Total Environ* 780:146274.
461 10.1016/j.scitotenv.2021.146274
462 Campbell Grant EH. 2008. Visual implant elastomer mark retention through metamorphosis in amphibian
463 larvae. *Journal of Wildlife Management* 72:1247-1252. 10.2193/2007-183
464 Chen S-C, Sun G-X, Rosen BP, Zhang S-Y, Deng Y, Zhu B-K, Rensing C, and Zhu Y-G. 2017. Recurrent
465 horizontal transfer of arsenite methyltransferase genes facilitated adaptation of life to arsenic. *Sci Rep*
466 7:7741. 10.1038/s41598-017-08313-2

Chen TH, Gross JA, and Karasov WH. 2009. Chronic exposure to pentavalent arsenic of larval leopard frogs (Rana pipiens): bioaccumulation and reduced swimming performance. *Ecotoxicology* 18:587-593. 10.1007/s10646-009-0316-3

Christian K, Weitzman C, Rose A, Kaestli M, and Gibb K. 2018. Ecological patterns in the skin microbiota of frogs from tropical Australia. *Ecol Evol* 8:10510-10519. 10.1002/ece3.4518

Cordeiro IF, Fonseca NP, Felestrino ÉB, Caneschi WL, Pires MRS, and Moreira LM. 2019. Arsenic resistance in cultured cutaneous microbiota is associated with anuran lifestyles in the Iron Quadrangle, Minas Gerais State, Brazil. *Herpetology Notes* 12: 1083-1093.

Costa RVF, Leite MGP, Mendonça FPC, and Nalini Jr. HA. 2015. Geochemical mapping of arsenic in surface waters and stream sediments of the Quadrilátero Ferrífero, Brazil. *Rem: Revista Escola de Minas* 68:43-51.

Crawley MJ. 2013. *The R book*: Second edition. Chichester, West Sussex, United Kingdom : Wiley, 2013.

Cruz LV. 2002. Avaliação Geoquímica Ambiental da Estação Ecológica do Tripuí e Adjacências, sudeste do Quadrilátero Ferrífero, Minas Gerais Mestrado em Evolução Crustal e Recursos Naturais Dissertação. Universidade Federal de Ouro Preto.

Daus B, Wennrich R, Morgenstern P, Weiß H, Leonhardt Palmieri HE, Nalini HA, Leonel LV, Guedes Monteiro RP, and Moreira RM. 2005. Arsenic Speciation in Plant Samples from the Iron Quadrangle, Minas Gerais, Brazil. *Microchimica Acta* 151:175-180. 10.1007/s00604-005-0397-5

FEMA. 1995. Plano de Manejo da Estação Ecológica do Tripuí, Ouro Preto - MG. Belo Horizonte: Fundação Estadual do Meio Ambiente - FEMA. p 71.

Flechas SV, Acosta-González A, Escobar LA, Kueneman JG, Sánchez-Quitian ZA, Parra-Giraldo CM, Rollins-Smith LA, Reinert LK, Vredenburg VT, Amézquita A, and Woodhams DC. 2019. Microbiota and skin defense peptides may facilitate coexistence of two sympatric Andean frog species with a lethal pathogen. *ISME J* 13:361-373. 10.1038/s41396-018-0284-9

Giguère A, Campbell PGC, Hare L, and Couture P. 2006. Sub-cellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (Perca flavescens) sampled along a polymetallic gradient. *Aquatic Toxicology* 77:178-189. https://doi.org/10.1016/j.aquatox.2005.12.001

Gupta SK, and Singh J. 2011. Evaluation of mollusc as sensitive indicatior of heavy metal pollution in aquatic system: A review.

Haddad CFB, Toledo LF, Prado CPA, Loebmann D, Gasparini JL, and Sazima I. 2013. *Guia dos Anfíbios da Mata Atlântica - Diversidade e Biologia*. São Paulo: Anolis Books Editora.

Hocking D, and Babbitt K. 2014. Amphibian contributions to ecosystem services. *Herpetological Conservation and Biology* 9:1-17.

Hopkins WA. 2007. Amphibians as Models for Studying Environmental Change. *ILAR Journal* 48:270-277. 10.1093/ilar.48.3.270

Jani AJ, and Briggs CJ. 2014. The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proceedings of the National Academy of Sciences* 111:E5049. 10.1073/pnas.1412752111

Johnson JH. 1971. Note on the validity of Machover's indicators of anxiety. *Percept Mot Skills* 33:126. 10.2466/pms.1971.33.1.126

Knutie SA, Wilkinson CL, Kohl KD, and Rohr JR. 2017. Early-life disruption of amphibian microbiota decreases later-life resistance to parasites. *Nat Commun* 8:86. 10.1038/s41467-017-00119-0

Kueneman JG, Parfrey LW, Woodhams DC, Archer HM, Knight R, and McKenzie VJ. 2014. The amphibian skin-associated microbiome across species, space and life history stages. *Mol Ecol* 23:1238-1250. 10.1111/mec.12510

Larsen EH. 2021. Dual skin functions in amphibian osmoregulation. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 253:110869. https://doi.org/10.1016/j.cbpa.2020.110869

Llewelyn VK, Berger L, and Glass BD. 2019. Permeability of frog skin to chemicals: effect of penetration enhancers. *Heliyon* 5:e02127. 10.1016/j.heliyon.2019.e02127

Luppi ASL, Santos ARd, Eugenio FC, and Feitosa LSA. 2015. Utilização de Geotecnologia para o Mapeamento de Áreas de Preservação Permanente no Município de João Neiva, ES. *Floresta e Ambiente* 22:13-22. https://doi.org/10.1590/2179-8087.0027

McKenzie VJ, Bowers RM, Fierer N, Knight R, and Lauber CL. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J* 6:588-596. 10.1038/ismej.2011.129

Miller WH, Jr., Schipper HM, Lee JS, Singer J, and Waxman S. 2002. Mechanisms of action of arsenic trioxide. *Cancer Res* 62:3893-3903.

Mohammed Abdul KS, Jayasinghe SS, Chandana EPS, Jayasumana C, and De Silva PMCS. 2015. Arsenic and human health effects: A review. *Environmental Toxicology and Pharmacology* 40:828-846. https://doi.org/10.1016/j.etap.2015.09.016

Moriarty MM, Koch I, and Reimer KJ. 2013. Arsenic species and uptake in amphibians (Rana clamitans and Bufo americanus). *Environmental Science: Processes & Impacts* 15:1520-1528. 10.1039/C3EM00223C

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, and Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403:853-858. https://doi.org/10.1038/35002501

Outten FW, Outten CE, and O'Halloran TV. 2000. Metalloregulatory Systems at the Interface Between Bacterial Metal Homeostasis and Resistance. In: Storz G, ed. *Bacterial Stress Responses*. Washington: ASM Press, 145-157.

Panceri Fleguer E, Rabelo Teixeira AF, and Costa de Melo AM. 2007. Caracterização preliminar da cacauicultura em João Neiva, Espírito Santo e sua relação com os princípios agroecológicos. *Revista Brasileira de Agroecologia* 2.

Pence NS, Larsen PB, Ebbs SD, Letham DLD, Lasat MM, Garvin DF, Eide D, and Kochian LV. 2000. The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator <em>Thlaspi caerulescens</em&gt. *Proceedings of the National Academy of Sciences* 97:4956. 10.1073/pnas.97.9.4956

Petrini R, Slejko F, Lutman A, Pison S, Franceschini G, Zini L, Italiano F, and Galic A. 2011. Natural arsenic contamination in waters from the Pesariis village, NE Italy. *Environmental Earth Sciences* 62:481-491. 10.1007/s12665-010-0541-3

Proença DN, Fasola E, Lopes I, and Morais PV. 2021. Characterization of the Skin Cultivable Microbiota Composition of the Frog Pelophylax perezi Inhabiting Different Environments. *International Journal of Environmental Research and Public Health* 18:2585.

Pyron RA. 2018. Global amphibian declines have winners and losers. *Proceedings of the National Academy of Sciences* 115:3739. 10.1073/pnas.1803477115

Rebollar EA, Gutierrez-Preciado A, Noecker C, Eng A, Hughey MC, Medina D, Walke JB, Borenstein E, Jensen RV, Belden LK, and Harris RN. 2018. The Skin Microbiome of the Neotropical Frog Craugastor fitzingeri: Inferring Potential Bacterial-Host-Pathogen Interactions From Metagenomic Data. *Front Microbiol* 9:466. 10.3389/fmicb.2018.00466

Rebollar EA, Hughey MC, Medina D, Harris RN, Ibáñez R, and Belden LK. 2016. Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of Batrachochytrium dendrobatidis. *ISME J* 10:1682-1695. 10.1038/ismej.2015.234

Reid MS, Hoy KS, Schofield JRM, Uppal JS, Lin Y, Lu X, Peng H, and Le XC. 2020. Arsenic speciation analysis: A review with an emphasis on chromatographic separations. *TrAC Trends in Analytical Chemistry* 123:115770. https://doi.org/10.1016/j.trac.2019.115770

Saha JC, Dikshit AK, Bandyopadhyay M, and Saha KC. 1999. A Review of Arsenic Poisoning and its Effects on Human Health. *Critical Reviews in Environmental Science and Technology* 29:281-313. 10.1080/10643389991259227

Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel A, Beukema W, Acevedo AA, Burrowes PA, Carvalho T, Catenazzi A, De la Riva I, Fisher MC, Flechas SV, Foster CN, Frias-Alvarez P, Garner TWJ, Gratwicke B, Guayasamin JM, Hirschfeld M, Kolby JE, Kosch TA, La Marca E, Lindenmayer

DB, Lips KR, Longo AV, Maneyro R, McDonald CA, Mendelson J, 3rd, Palacios-Rodriguez P, Parra-Olea G, Richards-Zawacki CL, Rodel MO, Rovito SM, Soto-Azat C, Toledo LF, Voyles J, Weldon C, Whitfield SM, Wilkinson M, Zamudio KR, and Canessa S. 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science* 363:1459-1463. 10.1126/science.aav0379

Schlebusch CM, Gattepaille LM, Engström K, Vahter M, Jakobsson M, and Broberg K. 2015. Human Adaptation to Arsenic-Rich Environments. *Molecular Biology and Evolution* 32:1544-1555. 10.1093/molbev/msv046

Team RC. 2021. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Trevelline BK, Fontaine SS, Hartup BK, and Kohl KD. 2019. Conservation biology needs a microbial renaissance: a call for the consideration of host-associated microbiota in wildlife management practices. *Proc Biol Sci* 286:20182448. 10.1098/rspb.2018.2448

Uren Webster TM, Bury N, van Aerle R, and Santos EM. 2013. Global Transcriptome Profiling Reveals Molecular Mechanisms of Metal Tolerance in a Chronically Exposed Wild Population of Brown Trout. *Environmental Science & Technology* 47:8869-8877. 10.1021/es401380p

Varejão EVV, Bellato CR, Fontes MPF, and Mello JWV. 2011. Arsenic and trace metals in river water and sediments from the southeast portion of the Iron Quadrangle, Brazil. *Environmental Monitoring and Assessment* 172:631-642. 10.1007/s10661-010-1361-3

Venturino A, Rosenbaum E, Caballero de Castro A, Anguiano OL, Gauna L, Fonovich de Schroeder T, and Pechen de D'Angelo AM. 2003. Biomarkers of effect in toads and frogs. *Biomarkers* 8:167-186. 10.1080/1354700031000120116

Walke Jenifer B, Becker Matthew H, Hughey Myra C, Swartwout Meredith C, Jensen Roderick V, Belden Lisa K, and Drake HL. 2015. Most of the Dominant Members of Amphibian Skin Bacterial Communities Can Be Readily Cultured. *Appl Environ Microbiol* 81:6589-6600. 10.1128/AEM.01486-15

Yao X-f, Zhang J-m, Tian L, and Guo J-h. 2017. The effect of heavy metal contamination on the bacterial community structure at Jiaozhou Bay, China. *Brazilian Journal Microbiology* 48:71-78. https://doi.org/10.1016/j.bjm.2016.09.007

Table

Table 1. General features of the species captured in the two study areas.

Figure captions

Figure 1. Collection regions of the investigated species. TES - Tripuí Ecological Station, an area naturally contaminated by metals, located in the state of Minas Gerais (yellow). JN – Municipality of João Neiva, uncontaminated area, located in the state of Espírito Santo (green). Images (a), (b), (c), (d), and (e) highlight the morphological characteristics of the five species collected and investigated in both regions.

Figure 2. Steps involved in the construction of the apparatus for simulating exposure to arsenic and analysis of the influence of skin-associated microbiota on the permeability of this contaminant. (A) Figurative summary of the steps described in the methodology. Numbers 1 to 7 represent the following steps: 1 – Reactivation of the selected bacteria; 2 – In parallel, fragments of bullfrog skin (*Lithobates catesbeianus*) were prepared; 3 –Skin fragments were placed in contact with bacterial cultures in Petri dishes and incubated at 28ºC for 24 hours; 4 – After incubation, skins were removed from the Petri dish and separated; 5 – The skins were tied to the open end of the test tube containing the arsenic solution (5 mM $NaAsO_2$ final concentration); 6 –Test tube containing the tied skin was poured into an Amphibian Ringer's solution (ARS); 7- Test tube was suspended by a rigid black paper plate, thus limiting the contact of the ARS with the environment. (B) Method for measuring the change in conductivity due to the permeabilization of arsenic through the amphibian skin, previously treated with bacteria.

Figure 3: Analysis of the tolerance of bacterial isolates to different concentrations of arsenic. (A) Evaluation of the degree of tolerance of bacterial isolates in different arsenic concentrations. n: total number of tolerant isolates in the investigated conditions. TES contaminated area and JN

uncontaminated area. (B) Evaluation of the degree of tolerance of bacterial isolates in the anuran species analyzed in the investigated areas. The numbers ranging from 0 to 100 represent the percentage of tolerance assessed at each of the six investigated growth times. The colors represent the arsenic concentrations to which the isolates were tolerant.

Figure 4. Qualitative analysis of the results obtained from the amphibian simulation apparatus to contaminated environments. (A) Growth chronology and tolerance profile of selected isolates at different arsenic concentrations. JN1 and JN2: isolates obtained from an uncontaminated area. TES1, TES2, and TES3: isolates obtained from contaminated area. The painted squares represent the times in which the growth rate of the isolates was confirmed in the respective arsenic concentrations (1mM yellow, 5 mM orange, and 10 mM red) (B) Change of conductivity in Amphibian Ringer's solution. The increase in conductivity is directly associated with the diffusion of arsenic through the amphibian skin. Error bars were determined from three independent experiments. (C) Summary of qualitative results associated with the use of the apparatus. + represents positive bacterial growth and – negative. Upward facing arrows indicate increased conductivity after 24 h of testing. The number of arrows is directly associated with the elevation of conductivity values.

Figure 5. The model proposed to suggest how the epithelial microbiota interferes in the adaptation of the anuran's species in contaminated environments. (A) Resilience (left) and animal susceptibility are dependent on the tolerance profile of the associated bacterial species. Species that have tolerant microbiota may not be good indicators of environmental contamination. (B) Metabolic diversity is found in bacteria associated with arsenic metabolism. 1 – Biofilm reduces metal contact with animal tissue; 2 – Redox reactions reduce ion toxicity; 3 – Biotransformation reactions transform more toxic species into less toxic ones; 4 – Efflux of ions or molecules carrying the contaminant; 5 – Chelation of harmful ions to organic molecules; 6 – Activation of oxidative species metabolism, increasing cell protection.

Supplemental files

Supplemental file 1. Raw data of empirical results. #isolates by specimen - spreadsheet that specifies the number of isolates for each of the 3 specimens of each species collected in the evaluated regions. #isolates by specie - spreadsheet that specifies the number of isolates for each species collected in the evaluated regions. As tolerance by specimen - spreadsheet that depicts the number of arsenic-tolerant isolates for each specimen at each of the evaluated concentrations. As tolerance assay by specie - spreadsheet that depicts the number of arsenic-tolerant isolates for each species in both regions evaluated. Condutivity assay - spreadsheet that identifies the conductivity values in the permeability tests of the anuran epithelial tissue to arsenic. Growth after permeability assay - spreadsheet that qualifies the growth profile of five isolates investigated after arsenic permeability assay to anuran epithelial tissue

Supplemental file 2: R script for statistical analysis.

Supplemental file 3: R input data analysis.

Supplemental file 4: R raw data output.

Table 1 (on next page)

Table 1 . General features of the species captured in the two study areas.

TES – Tripuí Ecological Station (contaminated area); JN – João Neiva (non-contaminated area).

1 **Table 1**. General features of the species captured in the two study areas.

2

Species	**Calling sites**	**Reproductive mode**	**Skin**	***n* specimens**		***n* isolates**	
				TES	JN	TES	JN
Boana faber	Inside nests built around the pond	Eggs placed directly in water	Smooth dorsal skin and slightly grainy ventral	3	3	60	64
Dendropsophus elegans	Around the edges of puddles and ponds that contain emergent, floating or herbaceous vegetation			3	3	86*	43
Hypsiboas albopunctatus			Smooth dorsal and ventral skin	3	3	58	48
Hypsiboas polytaenius	Tree trunks and leaves around the pond			3	3	39	69*
Rhinella crucifer	Around the pond		Dry and grainy skin on the belly and back	3	3	94	85
Total				**15**	**15**	**337**	**307**

3 TES – Tripuí Ecological Station (contaminated area); JN – João Neiva (non-contaminated area).

4

Figure 1

Figure 1. Collection regions of the investigated species.

TES - Tripuí Ecological Station, an area naturally contaminated by metals, located in the stateof Minas Gerais (yellow). JN – Municipality of João Neiva, uncontaminated area, located in the state of Espírito Santo (green). Images (a), (b), (c), (d), and (e) highlight the morphological characteristics of the five species collected and investigated in both regions.

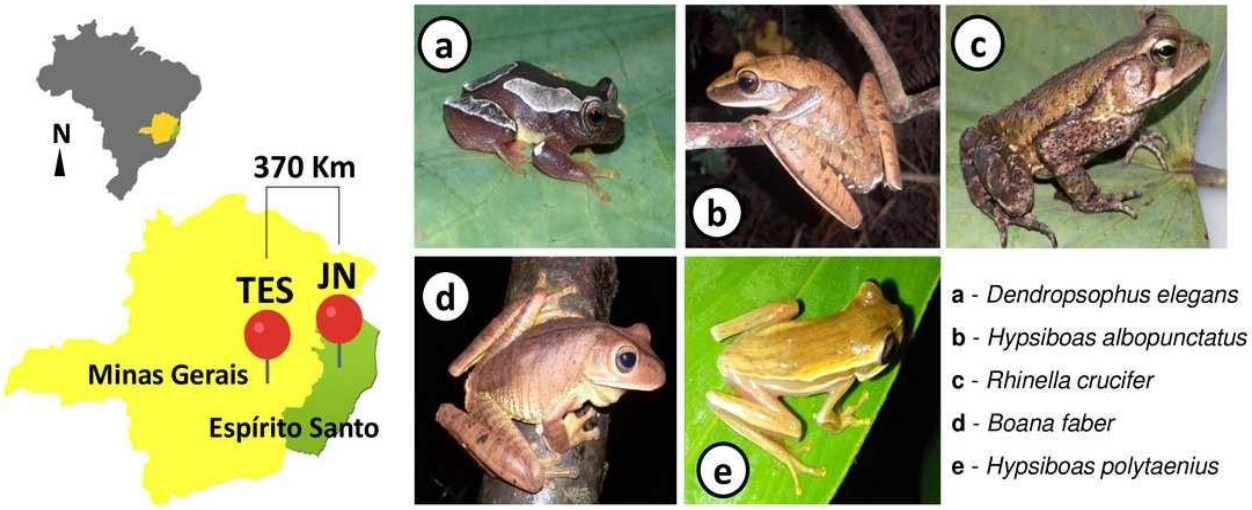


Figure 2

Figure 2. Steps involved in the construction of the apparatus for simulating exposure to arsenic and analysis of the influence of skin-associated microbiota on the permeability of this contaminant.

(A) Figurative summary of the steps described in the methodology. Numbers 1 to 7 representthe following steps: 1 – Reactivation of the selected bacteria; 2 – In parallel, fragments of bullfrog skin (Lithobates catesbeianus) were prepared; 3 –Skin fragments were placed in contact with bacterial cultures in Petri dishes and incubated at 28ºC for 24 hours; 4 – Afterincubation, skins were removed from the Petri dish and separated; 5 – The skins were tied to the open end of the test tube containing the arsenic solution (5 mM $NaAsO_2$ final

concentration); 6 –Test tube containing the tied skin was poured into an Amphibian Ringer'ssolution (ARS); 7- Test tube was suspended by a rigid black paper plate, thus limiting the contact of the ARS with the environment. (B) Method for measuring the change in conductivity due to the permeabilization of arsenic through the amphibian skin, previously treated with bacteria.

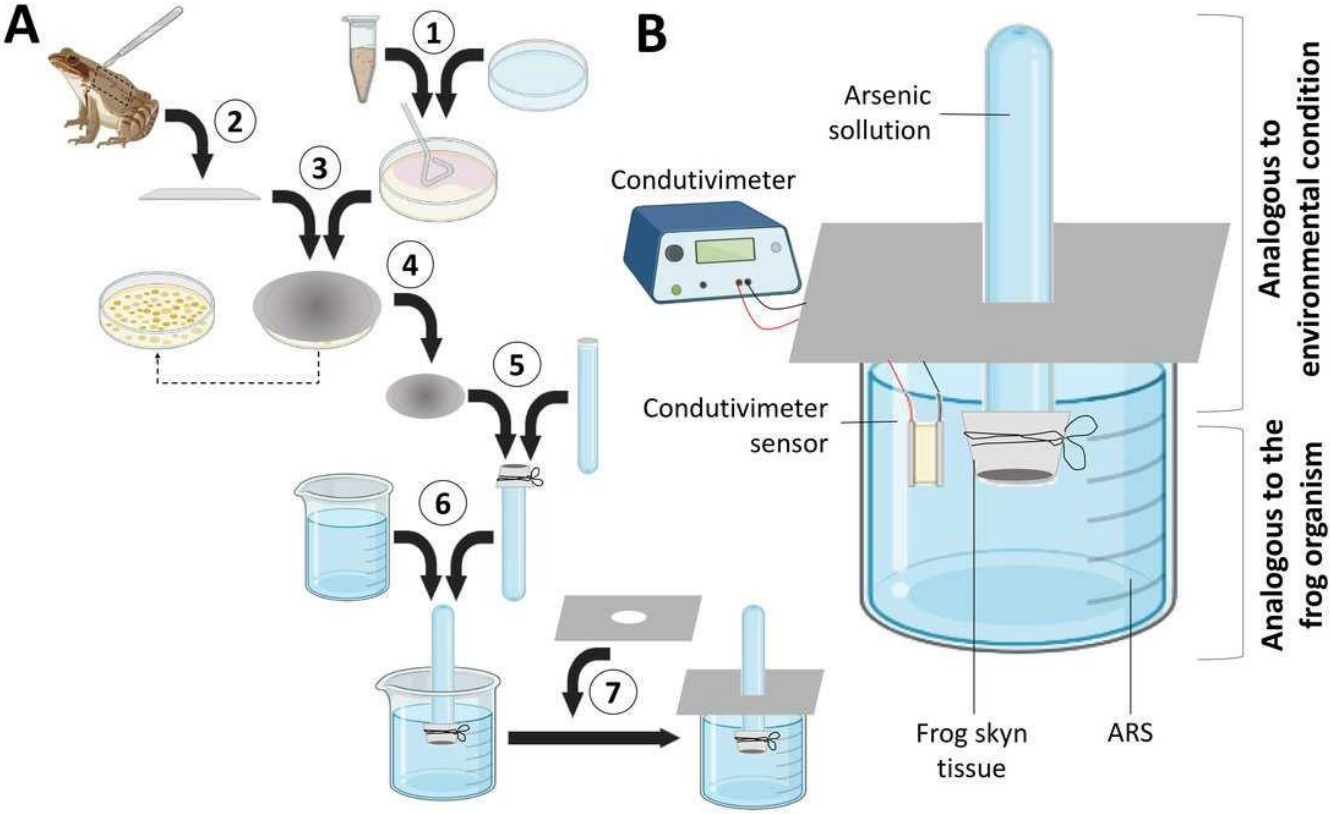

A
1
2
3
4
5
6
7
B
Arsenic
sollution
Condutivimeter
Condutivimeter
sensor
Frog skyn
tissue
ARS
Analogous to
environmental condition
Analogous to the
frog organism

Figure 3

Figure 3: Analysis of the tolerance of bacterial isolates to different concentrations of arsenic.

(A) Evaluation of the degree of tolerance of bacterial isolates in different arsenic concentrations. n: total number of tolerant isolates in the investigated conditions. TES contaminated area and JN uncontaminated area. (B) Evaluation of the degree of tolerance ofbacterial isolates in the anuran species analyzed in the investigated areas. The numbers ranging from 0 to 100 represent the percentage of tolerance assessed at each of the six investigated growth times. The colors represent the arsenic concentrations to which the isolates were tolerant.

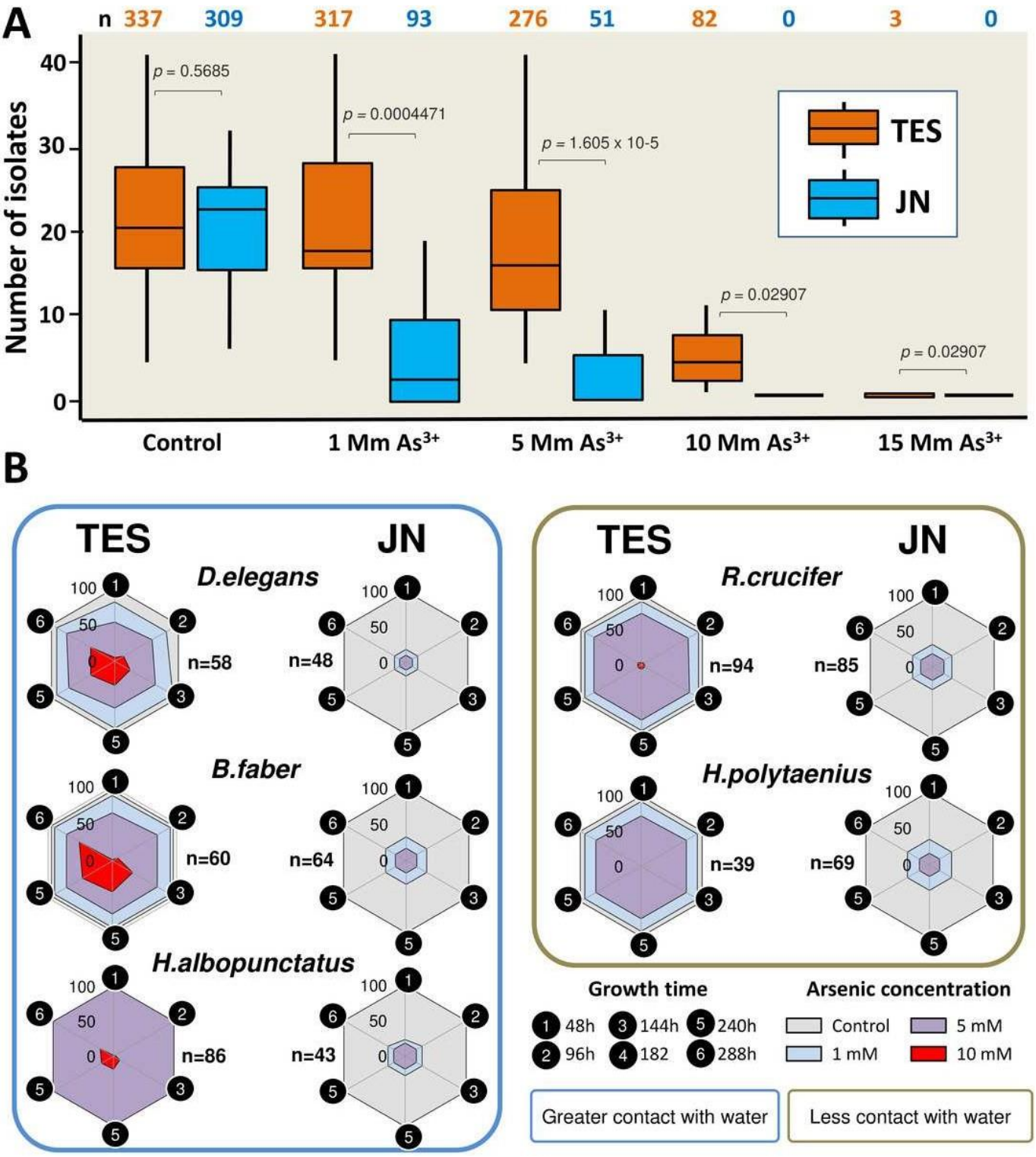

A
n 337 309 317 93 276 51 82 0 3 0
Number of isolates
40
30
20
10
0
p = 0.5685
p = 0.0004471
p = 1.605 x 10-5
p = 0.02907
p = 0.02907
TES
JN
Control
1 Mm As3+
5 Mm As3+
10 Mm As3+
15 Mm As3+
B
TES
JN
D.elegans
n=58
n=48
B.faber
n=60
n=64
H.albopunctatus
n=86
n=43
R.crucifer
n=94
n=85
H.polytaenius
n=39
n=69
Growth time
1 48h
2 96h
3 144h
4 182
5 240h
6 288h
Arsenic concentration
Control
1 mM
5 mM
10 mM
Greater contact with water
Less contact with water

Figure 4

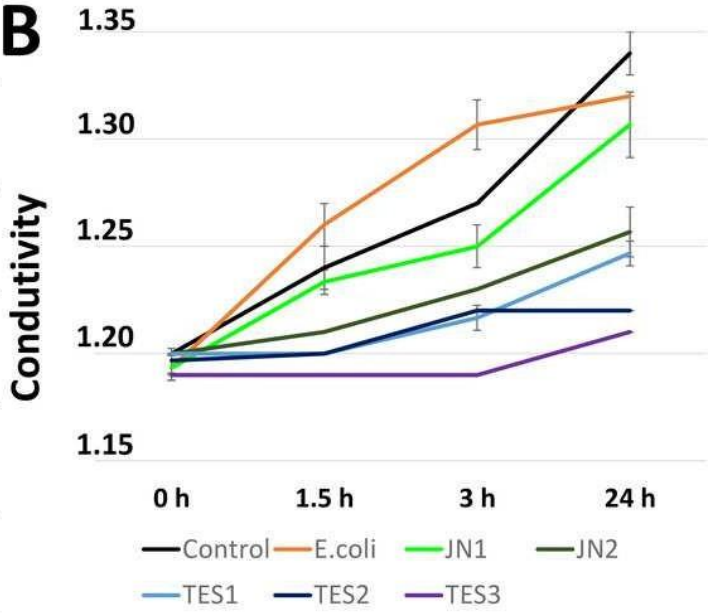
Figure 4. Qualitative analysis of the results obtained from the amphibian simulation apparatus to contaminated environments.

(A) Growth chronology and tolerance profile of selected isolates at different arsenic concentrations. JN1 and JN2: isolates obtained from an uncontaminated area. TES1, TES2, and TES3: isolates obtained from contaminated area. The painted squares represent the times in which the growth rate of the isolates was confirmed in the respective arsenic concentrations (1mM yellow, 5 mM orange, and 10 mM red) (B) Change of conductivity in Amphibian Ringer's solution. The increase in conductivity is directly associated with the diffusion of arsenic through the amphibian skin. Error bars were determined from three independent experiments. (C) Summary of qualitative results associated with the use of theapparatus. + represents positive bacterial growth and – negative. Upward facing arrows indicate increased conductivity after 24 h of testing. The number of arrows is directly associated with the elevation of conductivity values.

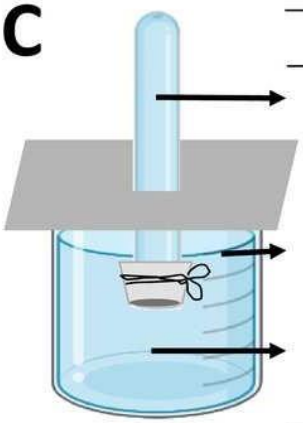
A

Frog host	Isolates	Hours 24 48 72 96	As^{3+} (mM)
H. polytaenius	JN1		1
			5
			10
B. faber	JN2		1
			5
			10
H. polytaenius	TES1		1
			5
			10
B. faber	TES2		1
			5
			10
H.albopunctatus	TES3		1
			5
			10

B



C

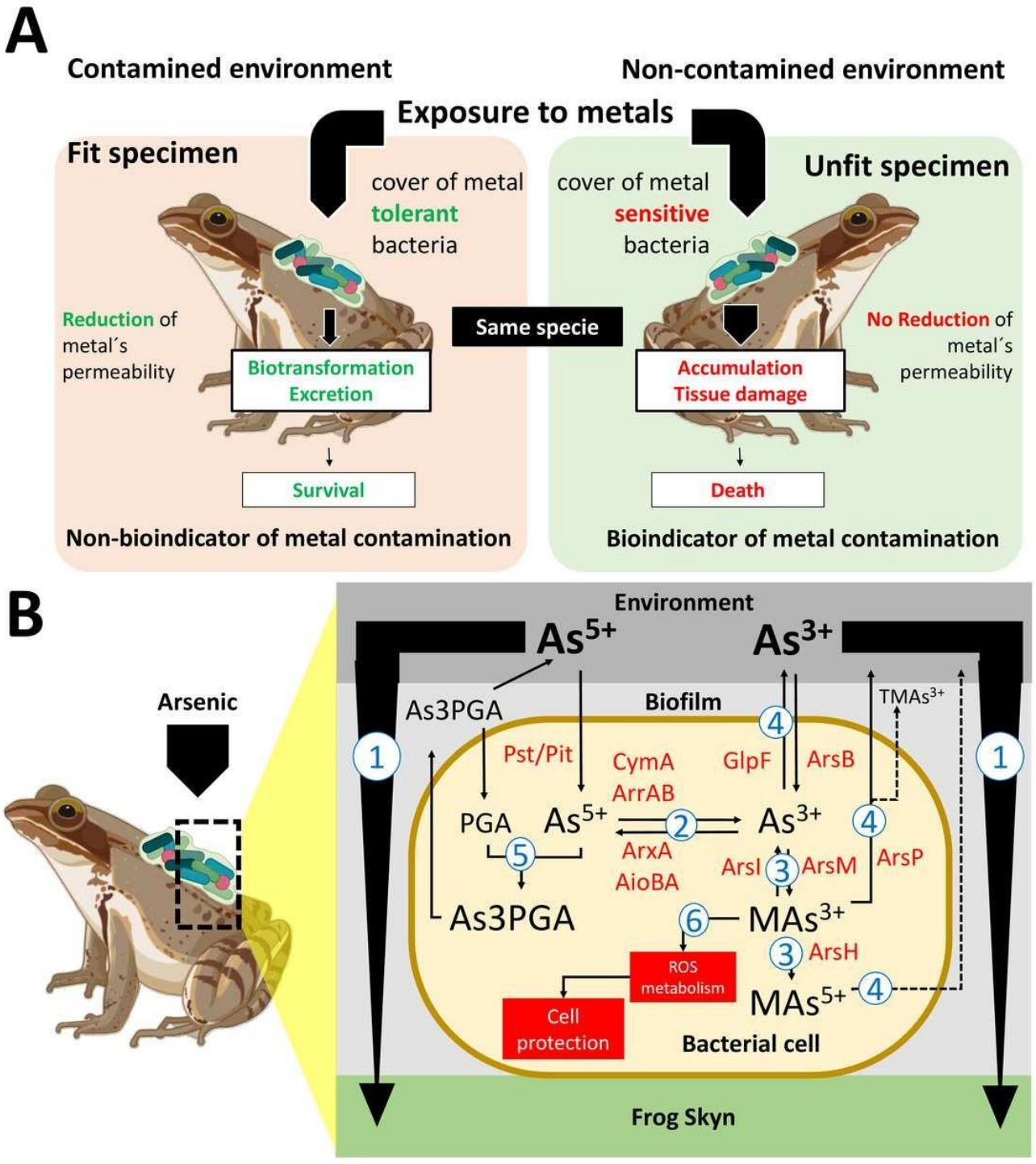


Parameters	Control	*E.coli*	JN 1	JN 2	TES 1	TES 2	TES3
Bac growth in the arsenic solution	-	-	-	-	+	+	+
Bac growth in the ARS	+	+	+	+	-	-	-
Condutivity in the ARS	↑↑↑	↑↑↑	↑↑	↑↑	↑↑	↑	↑

Figure 5

Figure 5. The model proposed to suggest how the epithelial microbiota interferes in theadaptation of the anuran's species in contaminated environments.

(A) Resilience (left) and animal susceptibility are dependent on the tolerance profile of the associated bacterial species. Species that have tolerant microbiota may not be good indicators of environmental contamination. (B) Metabolic diversity is found in bacteria associated with arsenic metabolism. 1 – Biofilm reduces metal contact with animal tissue; 2 –Redox reactions reduce ion toxicity; 3 – Biotransformation reactions transform more toxic species into less toxic ones; 4 – Efflux of ions or molecules carrying the contaminant; 5 – Chelation of harmful ions to organic molecules; 6 – Activation of oxidative species metabolism, increasing cell protection.


A
Contamined environment
Non-contamined environment
Exposure to metals
Fit specimen
cover of metal
tolerant
bacteria
Unfit specimen
cover of metal
sensitive
bacteria
Reduction of
metal´s
permeability
Biotransformation
Excretion
Same specie
No Reduction of
metal´s
permeability
Accumulation
Tissue damage
Survival
Death
Non-bioindicator of metal contamination
Bioindicator of metal contamination
B
Arsenic
Environment
As5+
As3+
As3PGA
Biofilm
TMAs3+
Pst/Pit
CymA
ArrAB
GlpF
ArsB
PGA
As5+
As3+
ArxA
AioBA
ArsI
ArsM
ArsP
As3PGA
MAs3+
ArsH
ROS
metabolism
MAs5+
Cell
protection
Bacterial cell
Frog Skyn

3.3- Capítulo III: Potencial biotecnológico de bactérias associadas ao tecido cutâneo de anuros da Estação Ecológica do Tripuí, Quadrilátero Ferrífero, MG.

Este capítulo é resultado da busca de possíveis potenciais biotecnológicos a partir de uma triagem realizada no banco de isolados formado ao longo do desenvolvimento dos estudos anteriormente descritos. Devido as restrições impostas pelo período de isolamento da pandemia, muitos ensaios foram inviabilizados, comprometendo o desenvolvimento do estudo para que fosse possível a publicação de um terceiro artigo referente a pesquisa desenvolvida.

Em resumo, o avanço da contaminação ambiental por metais potencialmente tóxicos demonstra a importância de se buscar por tecnologias sustentáveis. Dessa forma, diversos estudos com o intuito de minimizar ou remover resíduos tóxicos do meio ambiente investigam a possível aplicação de microrganismos no processo de descontaminação dos ambientes. Para esse estudo, foi selecionado 1 isolado proveniente de um banco de 1251 isolados formado a partir de estudos anteriores que investigavam a tolerância da microbiota do tecido cutâneo de anuros em uma região do Quadrilátero Ferrífero contaminada por arsênio. Os resultados apresentados neste capítulo expõem possíveis aplicações biotecnológicas do isolado, seja devido à alta tolerância a distintos metais, ou pela característica de alterar a cor em diferentes concentrações.

1. Introdução

Os organismos vivos estão constantemente expostos a fatores que podem comprometer sua integridade física (Ahmann et al., 1994). Particularmente, os anfíbios representam um grupo de vertebrados que são mais vulneráveis a condições do meio, já que possuem uma pele altamente permeável, que pode permitir a passagem livre de agentes químicos e biológicos do ambiente (Eterovick et al., 2005). A superfície cutânea desses animais apresenta atributos únicos dentre os vertebrados e atua como uma interface bastante funcional entre o organismo e o meio externo (Eterovick et al., 2005). Para além da proteção mecânica, a pele inclui mecanismos bioquímicos e biológicos derivados da comunidade microbiana e da secreção de moléculas bioativas (Louise et al., 2005).

Dessa forma, o entendimento da funcionalidade de proteção que a comunidade microbiana oferece para o animal é importante, uma vez que, a partir do estudo de interações ecológicas, podem ser descobertos possíveis organismos com potenciais aplicações biotecnológicas. Nessa linha, em estudo preliminar (Cordeiro et al., 2019), foi investigada a influencia do ambiente na composição da microbiota cutânea de anfíbios anuros em uma região contaminada arsênio, bem como o papel de tal comunidade na proteção do anfíbio em ambiente com características hostis. Os resultados indicaram que o maior número de isolados bacterianos, do mesmo modo, a maior resistência ao arsênio e a maior capacidade de produção de biofilme, foram associados a espécies de anuros que apresentavam maior contato com o arsênio presente na água durante o período reprodutivo. O arsênio é um metaloide, o qual, apresenta ampla distribuição sendo encontrado desde solos até organismos, e se encontra mais disponível na água (Wang e Zhao, 2009). Logo, os resultados auxiliaram a melhor compreensão desse modelo de interação, bem como classificam potenciais isolados com capacidades biotecnológicas.

A resistência de isolados bacterianos em meios contaminados por metais pesados e metaloides já foi constatada em distintos estudos, como o de Nies (2000), que discutiu sobre a funcionalidade das bactérias como ferramentas para a avaliação e remediação de ambientes contaminados. Uma vez que, em estudo preliminar desenvolvido por nossa equipe anteriormente citado (Cordeiro et al., 2019), foi constatado a resistência de isolados bacterianos ao arsênio, bem como a alteração de cor de alguns isolados quando expostos ao metaloide, o objetivo do presente estudo foi investigar os mecanismos associados a resistência e a alteração de cor, assim como os possíveis potenciais biotecnológicos de aplicação dessas características.

2. Materiais e métodos

a. Seleção do isolado

A partir de estudos anteriores desenvolvidos ao longo do mestrado e que foram continuados no doutorado, formou-se um banco com 1251 isolados bacterianos que foram preservados em eppendorf de 1,5 mL. Esses isolados foram expostos a concentrações crescentes de arsênio e desse número inicial de 1251 isolados, 5 apresentaram alteração de cor quando expostos ao metaloide.

Os isolados bacterianos previamente selecionados e preservados foram transferidos para placa de 96 poços contendo 100 µL de meio LB líquido em cada poço, foi utilizada uma placa de 96 poços para cada grupo. Cada isolado foi colocado em um poço. As placas foram incubadas por 48 h a 28°C para o crescimento dos isolados. Nessas placas, dois poços foram preenchidos apenas com meio líquido, sem nenhum isolado, representando o controle negativo para o teste. Ou seja, nas devidas posições destes poços não haverá crescimento e dois poços são preenchidos com um isolado que apresenta crescimento comprovado nas condições dos testes, representando o controle positivo. Para cada placa os 96 poços são mapeados, de modo a permitir a identificação inequívoca do isolado que se encontra em cada poço, bem como a localização dos controles negativos e positivos.

Após a transferência para as placas de 96 poços, os isolados foram novamente expostos a concentrações menores de arsênio para avaliar a sensibilidade da alteração da cor, esta análise permitiu a seleção de 1 isolado que apresentou perda gradativa da cor quando exposto a diluições menores de arsênio e identificado como G1. Para tanto, foram utilizadas placas de Petri com meio LB sólido com concentrações diluídas de As 3+ (0,1mM; 0,2mM; 0,3mM; 0,4mM; 0,5mM; 0,6mM; 0,7mM; 0,8mM; 0,9mM; 1Mm) e a placa controle contendo somente meio LB sólido. Com o auxílio do replicador, os isolados presentes na placa de 96 poços foram transferidos para placas de Petri identificadas e incubados a 28°C, fazendo uso de um multireplicador de 96 pontas. Registros fotográficos foram realizados em intervalos de 24hrs ao longo de 3 dias para acompanhar o crescimento dos isolados.

b. Caracterização da melhor condição de crescimento

Para estabelecer a melhor condição de crescimento, foram avaliadas diferentes variáveis como: meio, pH e temperatura, no intuito de observar em quais parâmetros o isolado mantinha a cor inicial quando foi selecionado para o estudo. Inicialmente, foi testado diferentes meios para inoculação do isolado. A partir desse primeiro ensaio, adotou-se o meio rico, Luria-

Bertani (LB), composto por bacto-tryptone (peptona), bacto-yeast extract (extrato de levedura), NaCl e água destilada. Em um segundo ensaio, foram avaliados diferentes valores de pH e temperatura, e para essas variáveis foram adotados os de valores de 5 e 28°C respectivamente.

c. Capacidade de resistência

O isolado G1foi exposto a diversos elementos em diferentes concentrações. Para isso, foram utilizadas placas de Petri com meio LB sólido com concentrações distintas de alumínio, arsenito, arsenato, cádmio, cobre, chumbo, ferro, manganês níquel, e zinco (1mM, 5mM, 10mM e 15mM) e a placa controle contendo somente meio LB sólido. O isolado preservado foi transferido para tubo contendo meio LB líquido e incubado por 24 horas a 28°C, após foi colocado em OD1 e transferido 10 mililitros para as placas de Petri identificadas e incubadas a 28°C. Registros fotográficos foram realizados em intervalos de 24hrs ao longo de 3 dias para acompanhar o crescimento dos isolados.

d. Alteração de cor

O ensaio de alteração de cor foi realizado através de placas de Petri com meio LB sólido com concentrações distintas de alumínio, arsenito, arsenato, cádmio, cobre, chumbo, ferro, manganês níquel, e zinco (1mM, 2mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, e 10mM) e a placa controle contendo somente meio LB sólido. O isolado preservado foi transferido para tubo contendo meio LB líquido e incubado por 24 horas a 28°C, após foi colocado em OD1 e transferido 10 mililitros para as placas de Petri identificadas e incubadas a 28°C. Registros fotográficos foram realizados em intervalos de 24hrs ao longo de 3 dias para acompanhar o crescimento dos isolados. Após a realização do ensaio de exposição a diferentes metais, foi realizado um segundo ensaio com a mesma metodologia descrita anteriormente, na presença dos metais em que houve alteração da cor, porém para esse ensaio o isolado foi exposto a diferentes diluições do metal em que apresentou variação da cor.

e. Motilidade

Para avaliar a capacidade de motilidade, foram utilizadas placas de Petri com meio LB semi sólido (0,3%) com concentrações distintas de alumínio, arsenito, arsenato, cádmio, cobre, chumbo, ferro, manganês níquel, e zinco (1mM, 2mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, e 10mM) e a placa controle contendo somente meio LB semi sólido (0,3%). O isolado preservado foi transferido para tubo contendo meio LB líquido e incubado

por 24 horas a 28°C, após foi colocado em OD1 e transferido 10 mililitros para as placas de Petri identificadas e incubadas a 28°C. Após 24 h de incubação a 28°C, as placas foram avaliadas e os diâmetros das respectivas colônias foram medidos. Para medir o diâmetro, utilizou-se a média de duas réplicas experimentais dos resultados obtidos através da operação: maior medida do eixo x multiplicado pela maior medida do eixo y em milímetro.

f. Produção de biofilme

A capacidade de produção de biofilme foi baseado no protocolo O'Toole et al (2000), com as adaptações descritas abaixo. O isolado preservado foi transferido para tubo contendo meio LB líquido e incubado por 24 horas a 28°C, após foi colocado em OD1 e transferido 10 mililitros e transferido em duplicata para placas de 96 poços contendo meio LB líquido, com concentrações distintas de alumínio, arsenito, arsenato, cádmio, cobre, chumbo, ferro, manganês níquel, e zinco (1mM, 5mM, 10mM) e incubado em 28 ° C por 24 h. Após a incubação, cada placa foi lavada duas vezes com água destilada. Depois de 1 h de secagem à temperatura ambiente (21-24°C), 125 µl de solução de violeta de cristal (10% g / v) foram adicionados a cada um dos 96 poços, e aguardou-se 45min. Após 1 h de secagem à temperatura ambiente (21–24°C), outra lavagem com água destilada foi realizada, seguido pela adição de 125 µl de etanol a 95% e aguardou-se mais 45 min. Logo, as soluções dos poços foram avaliadas usando um Espectrofotômetro Victor X3 (Perkin Elmer, Waltham, Massachusetts, EUA) em um comprimento de onda de 550 nm para medir a absorbância. Para este ensaio, poços contendo apenas o meio LB líquido foram considerados negativo controles, enquanto o controle positivo tinha conhecido produtores de biofilme no respectivo poço. Com base no valores de absorbância obtidos, os isolados bacterianos foram classificados arbitrariamente em três categorias, calculando a média de duas réplicas experimentais: (1) não produção de biofilme (OD $\leq$ 0,19); (2) produção moderada (OD entre 0,20 e 0,30); e (3) alta produção de biofilme (OD $\geq$ 0,31).

3. Resultados

a. Capacidade de resistência

A análise de resistência a outros metais demonstrou a resistência do isolado selecionado (G1) a dez metais: alumínio, arsenato, arsenito, cádmio, cobre, chumbo, ferro, manganês, níquel, zinco. A concentração de resistência para cada metal foi descrita na quadro 1.

Quadro 1: Resistência do isolado G1 a diferentes metais e concentrações. As células em cinza representam a resistência do isolado a determinada concentração.

	controle	1mM	2mM	3mM	4mM	5mM	6mM	7mM	8mM
Arsênio									
Cádmio									
Arsenato									
Zinco									
Níquel									
Ferro									
Chumbo									
Cobre									
Alumínio									
Manganês									

b. Alteração da cor

No ensaio de alteração de cor, o isolado apresentou variação quando exposto a cinco metais: arsenato, arsenito, cádmio, cobre e zinco. Tal resultado foi demonstrado na figura 2. No ensaio posterior com as diluições dos cinco metais em que houve alteração da cor, o isolado apresentou variação em apenas três: arsenato, arsenito e cádmio (Figura 3).

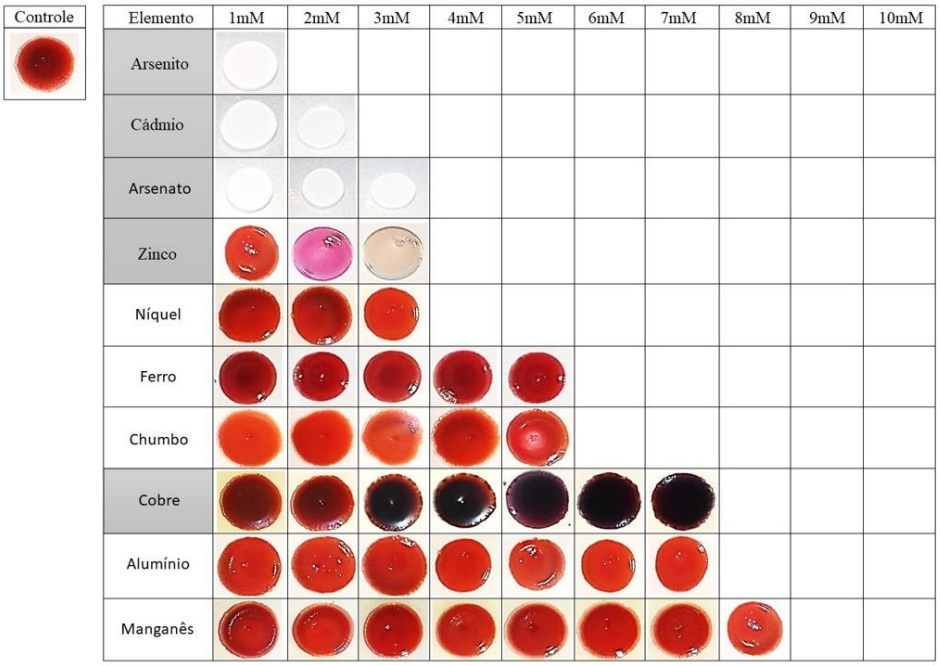


Figura 2: Foto do crescimento do isolado nas placas de Petri com as concentrações dos diferentes elementos. Resultado em 48 horas após inoculação de 10 mililitros do isolado. A esquerda da imagem, crescimento do isolado na placa controle contendo somente meio LB e nenhum elemento, a direita da imagem, resultado do crescimento nas placas com os diferentes elementos e concentrações.

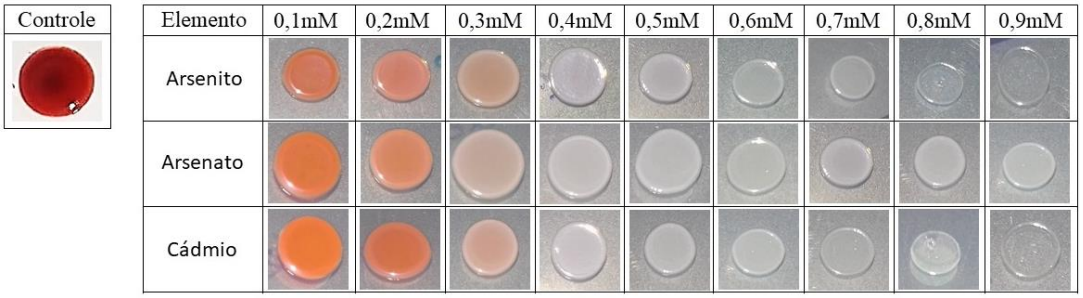


Figura 3: Resultado do ensaio de exposição do isolado as diferentes diluições em 48 horas. A esquerda da imagem, crescimento do isolado na placa controle contendo somente meio LB nenhum elemento, a direita da imagem, resultado do crescimento nas placas com os diferentes elementos e diluições.

c. Motilidade

O ensaio de motilidade demonstrou que com o aumento da concentração houve uma redução na motilidade. Tal resultado foi observado em todos os metais e pode ser visto na figura 4 e no gráfico 1.

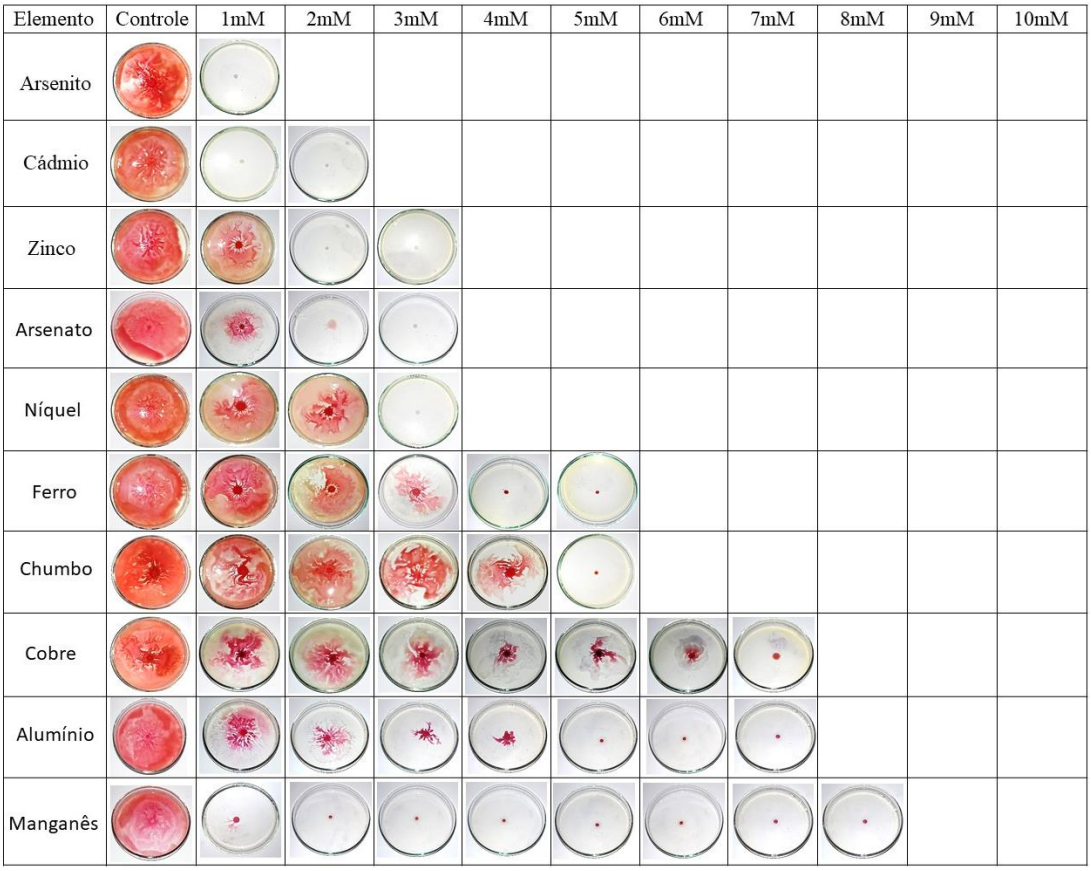


Figura 4: Resultado do ensaio de motilidade quando o isolado foi exposto em diferentes concentrações em 24 horas. A esquerda da imagem, crescimento do isolado na placa controle contendo somente meio LB e nenhum metal, a direita da imagem, resultado do crescimento nas placas com as diferentes concentrações.

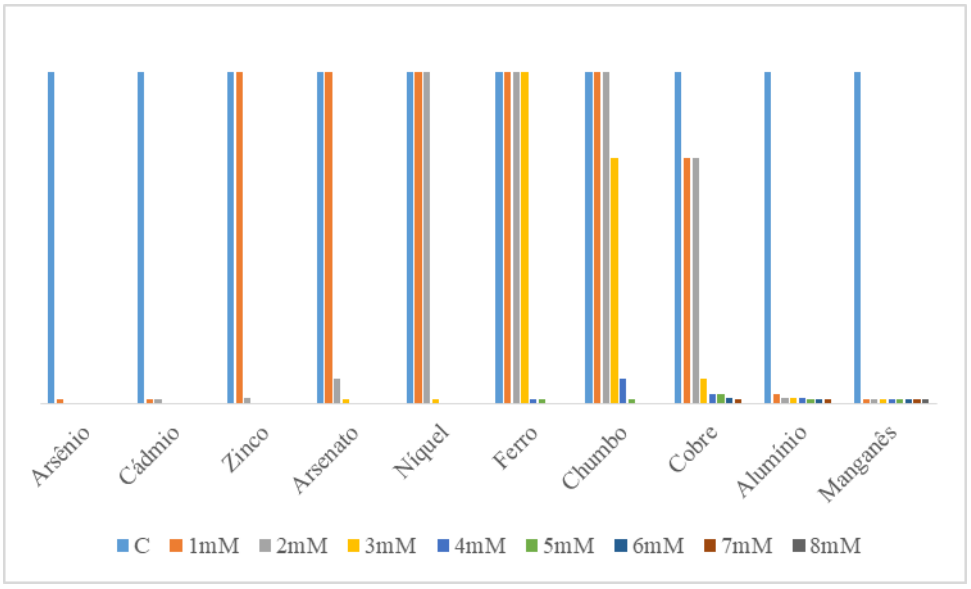


Gráfico 1: Dados da motilidade nas diferentes concentrações dos metais (controle, 1mM, 2mM, 3mM, 4mM, 5mM, 6mM, 7mM e 8Mm), medidos em milímetro.

d. Produção de biofilme

O teste que avaliou a produção de biofilme demonstrou que com o aumento da concentração, houve um aumento na produção de biofilme. O resultado de proporcionalidade do aumento da produção com o aumento da concentração foi observado em todos os metais (Gráfico 2).

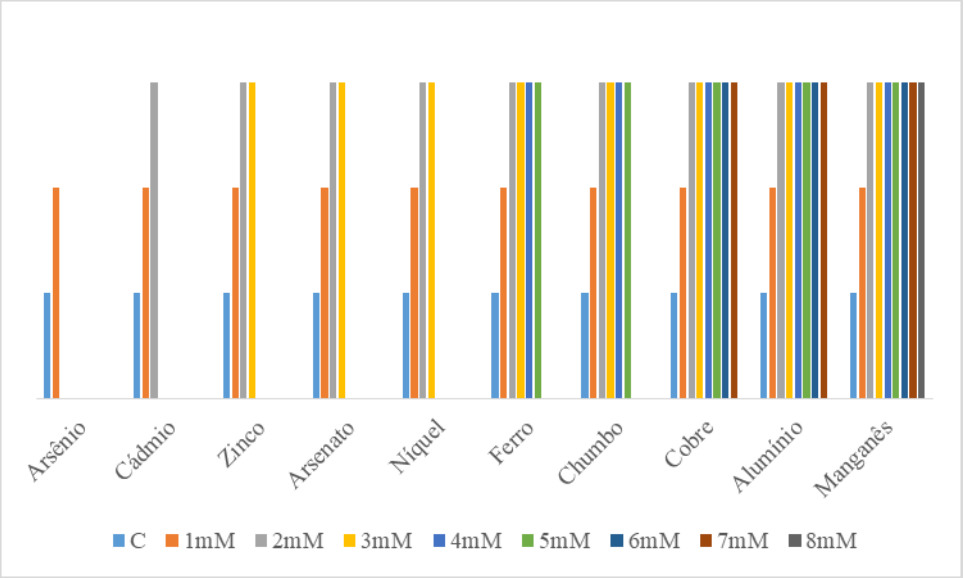


Gráfico 2: Representação do resultado da produção de biofilme nas diferentes concentrações para cada metal (controle, 1mM, 2mM, 3mM, 4mM, 5mM, 6mM, 7mM e 8Mm).

4. Discussão

A degradação dos recursos naturais e a contaminação do ambiente são crescentes e promovem graves consequências para o meio (Masindi e Khathutshelo, 2018). Uma das formas de contaminação ambiental com grande relevância no Brasil, é a presença de metais encontrados em altas quantidades no meio (Borba e Figueiredo, 2004). Os metais podem ser encontrados naturalmente no ambiente, no entanto, a emissão pelo homem através da mineração, queima de carvão e outros processos industriais agravam a contaminação, são mais nocivos aos organismos vivos e seus resíduos podem se tornar um risco para todo o ecossistema (Borba e Figueiredo, 2004).

Nesse sentido, o aumento contínuo da degradação ambiental fez com que a busca por métodos de avaliação dessa degradação também aumentasse, sobretudo técnicas e estudos com seres vivos capazes de diferenciar as oscilações do ambiente em um determinado tempo. Tais seres vivos respondem por meio de reações comportamentais ou metabólicas mensuráveis e indicam mudanças no habitat onde vivem (Markert et al., 2003). Os seres caracterizados como bioindicadores estão presentes nos variados níveis de organização biológica (Markert et al., 2003).

Microrganismos, muitas vezes, são utilizados como bioindicadores devido à sensibilidade em relação as variáveis físicas e químicas do ambiente (Astudillo et al., 2019). Dentre a empregabilidade dos microrganismos se destacam a caracterização do solo e da água através da presença ou ausência de determinadas comunidades de microrganismos (Ndiaye e Dick, 2000). As variações nas populações microbianas ocorrem sobretudo em decorrência de modificações do pH, da umidade, da aeração, da temperatura e da disponibilidade de elementos orgânicos e inorgânicos (Giller e Mcgrath, 1998).

O uso de bioindicadores envolve avaliações múltiplas de parâmetros a respeito da atividade biológica (Clements, 2000). Dessa forma, os resultados obtidos com o presente estudo permitem inferir que o isolado avaliado apresenta capacidade de utilização como bioindicador da presença de determinados metais no meio. Isso se dá devido ao isolado apresentar resistência a 10 metais e dentre esses, possui alteração da cor em 4: arsenato, arsenito, cádmio e zinco, sendo que para os 3 primeiros, a alteração de cor apresentou uma sensibilidade maior com a diluição da solução de 1mM.

Segundo a literatura (Ron, 2006), a resposta ao estresse bacteriano permite que esses organismos sobrevivam a condições adversas e flutuantes em seu entorno, e tal resposta envolve vários mecanismos que reconhecem alterações ambientais e formam uma resposta

favorável. As bactérias podem reagir simultaneamente a uma gama de estresse e os sistemas de resposta envolvem expressão gênica, atividades proteicas, redes regulatórias, bem como a morfologia celular (Navarro et al., 2013). A alteração de cor encontrada no desenvolvimento do estudo pode estar associada a reações químicas que fazem parte do sistema de resposta ao estresse quando na presença de metais. Assim, a investigação dessas respostas é importante na caracterização do microrganismo como potencial bioindicador.

Nesse sentido, a capacidade de produção de biofilme faz parte do mecanismo de resposta ao estresse. Nesse processo, a bactéria secreta substâncias poliméricas extracelulares para formar um filme formado por comunidades de células agregadas, organizadas e funcionais, capaz de fornecer além de suporte, proteção para condições estressantes (Barros et al., 2014). Os resultados do ensaio referente a capacidade de produção de biofilme demonstraram que com o aumento da concentração dos metais, houve um aumento na produção do biofilme. Logo, o biofilme representa um modo de crescimento que permite que o isolado sobreviva na presença dos metais e contribui para tornar o isolado substancialmente mais resistente.

Em bactérias, outro importante sistema de resposta ao estresse é a motilidade. A motilidade bacteriana faz parte da variedade de mecanismos de mobilidade para explorar os recursos e ambientes disponíveis, do mesmo modo, permite que as bactérias se afastem de ambientes estressantes (Bizani e Souza, 2016). No entanto, os resultados do estudo mostraram uma diminuição da motilidade com o aumento da concentração dos metais, o que permite inferir que outros mecanismos conferem resistência ao isolado, fazendo com que seja tolerante a presença dos metais e não use a motilidade como escape da condição integrante já que apresenta tolerância.

Por fim, bioindicadores podem ser classificados quanto sua sensibilidade e resposta ao ambiente, bem como podem ser divididos em indicadores ambientais e indicadores ecológicos (McGeoch, 1998). A primeira classificação diz respeito ao grupo que responde de forma previsível às perturbações ambientais, já a segunda representa as espécies consideradas sensíveis a alterações do meio. Diante do exposto, o presente estudo evidenciou a possível empregabilidade de um isolado bacteriano prospectado do tecido cutâneo de anuro, bem como as características ideias para sua utilização. Além disso, o estudo buscou fazer uma breve discussão sobre outros possíveis usos de organismos como bioindicadores.

5. Conclusão

A crescente demanda de tecnologias e produtos ecologicamente corretos faz com que o uso de organismos vivos como bioindicadores de qualidade ambiental apresente-se como uma das novas táticas para o monitoramento e avaliação do ambiente. Neste trabalho, foi possível avaliar um isolado prospectado do tecido cutâneo de anuro em local contaminado por metais, em relação a capacidade de uso como bioindicador de locais contaminados. Assim, a alteração de cor na presença dos determinados metais demonstra que o uso de bioindicadores é muito mais vasta que o exposto na literatura, e o reconhecimento dessa amplitude pode subsidiar outras pesquisas.

REFERÊNCIAS

Abdelatey, L. M. et al. Heavy metal resistance and gene expression analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Egyptian soils. J. Appl. Sci. Environ. Sanitation v. 6, n. 2, p. 201-211, (2011).

Ahmann, D., Roberts, A. L., Krumholz, L. R., & Morel, F. M.. Microbe grows by reducing arsenic. Nature, 371(6500), 750. (1994).

Assis, A. B. Microbiota, secreções cutâneas e microclima: consequências para os anfíbios. Revista da Biologia, v. 8. p. 45-48. (2012).

Astudillo-García, C., Hermans, S.M., Stevenson, B. et al. Microbial assemblages and bioindicators as proxies for ecosystem health status: potential and limitations. Appl Microbiol Biotechnol 103, 6407–6421 https://doi.org/10.1007/s00253-019-09963-0.(2019).

Barbosa, Getúlio V.; Rodrigues, David Márcio dos Santos. Quadrilátero Ferrífero. Belo Horizonte: Universidade Federal de Minas Gerais, 130p. (1967).

Barros, André Luiz Raposo, et al. "resistência a metais pesados, antimicrobianos e formação de biofilme em cepas de escherichia coli isoladas de praias de São Luis-maranhão." Revista de Patologia Tropical/Journal of Tropical Pathology 43.3 277-289. (2014).

Bertin, P. N., Heinrich-Salmeron, A., Pelletier, E., Goulhen-Chollet, F., Arsène-Ploetze, F., Gallien, S., & Bonnefoy, V. Metabolic diversity 35 among main microorganisms inside an arsenic-rich ecosystem revealed by meta-and proteo-genomics. The ISME journal, 5(11), 1735-1747. (2011).

Bizani, Delmar, and Matheus Souza Spagiari. "Avaliação da cinética de comportamento e capacidade de redução de cromo por linhagens bacterianas padrão." Ciência e Natura 38.2: 648-655. (2016).

Borba, R. P. Arsênio em ambiente superficial: processos geoquímicos naturais e antropogênico em uma área de mineração aurífera. Tese de doutorado, Instituto de Geociências, Universidade Estadual de Campinas, 202p. (2002).

Borba, R.P., & Figueiredo, B. R. A influência das condições geoquímicas na oxidação da arsenopirita e na mobilidade do arsênio em ambientes superficiais tropicais. Rev Bras Geociências 34:489–500. (2004).

Clements WH. Integrating effects of contaminats across levels of biological organization. J. Ecosy Stress and Rec ; 7:113-116. (2000).

Colombo, P.; Zank, C. Biodiversidade dos Campos de Cima da Serra. Porto Alegre:Libretos, (2008).

Cordeiro, I. F.; Fonseca, N. P.; Felestrino, É. B.; et al. Arsenic resistance in cultured cutaneous microbiota is associated with anuran lifestyles in the iron quadrangle, Minas Gerais State, Brazil. Herpetology Notes, v. 12, n. October, p. 1083–1093, (2019).

Crespo, J. C. M. Reflexões sobre a patrimonialização das paisagens no Quadrilátero Ferrífero de Minas Gerais, MG. Sessão temática: Patrimônio, território e paisagem cultural: novos desafios à preservação. Encontro da Associação Nacional de Pesquisa e Pós-Graduação em Arquitetura e Urbanismo. Porto Alegre (2016).

Cruz CAG and Feio RN. Endemismos em anfíbios em áreas de altitude na Mata Atlântica no sudeste do Brasil. In: Nascimento LB and Oliveira ME (Eds), Herpetologia no Brasil II. Soc Bras Herpet, p. 117-126. (2007).

Dembitsky, V. M., & Rekanza, T. Natural Occurrence of Arseno Compounds in Plants, Lichens, Fungi, Algal Species, and Microorganismos. Plant Science, 165, 1177-1192. (2003).

Drummond, G. M., Martins, C. S., Machado, A. B. M., Sebaio, F. A., Antonini, Y. Biodiversidade em Minas Gerais: um atlas para a sua conservação. Belo Horizonte: Fundação Biodiversitas. 222p. (2005).

Eterovick, P. C., Carnaval, A. C. O. Q., Borges-nojosa, D. M., Silvano, D. L., Segalla,

M. V., & Sazima, I. Amphibian declines in Brazil: an overview. Biotropica 37(2): 166-179. (2005).

Feder, M. E.; Burggren, W. W. Environmen tal physiology of the amphibians. Chicago: The University of Chigago Press, (1992).

Gause, G. F. “Experimental Populations of Microscopic Organisms.” Ecology, vol. 18, no. 2, pp. 173–179. JSTOR, (1937).

Giller, K. E., Witter, E., & Mcgrath, S. P. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology and Biochemistry, 30(10), 1389-1414. (1998).

Hacioglu, N., & Tosunoglu, M. Determination of antimicrobial and heavy metal resistance profiles of some bacteria isolated from aquatic amphibian and reptile species. Environmental monitoring and assessment, 186(1), 407-413. (2014).

Hall, J. L. Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot, v. 53, n. 366, p. 1-11, (2002).

Harris, R. N., James, T.Y., Lauer, A., Simon, M.A., & Patel, A. Amphibian pathogen Batrachochytrium dendrobatidis is inhibited by the cutaneous bacteria of amphibian species. EcoHealth, 3, 53-56. (2006).

Instituto Prístino: Atlas Digital Geoambiental . Sistema WebGis de livre acesso ao banco de dados ambiental. Disponível em: < https://institutopristino.org.br/atlas/>.Acesso em: 07/02/2022.

Jacobi, C. M., Carmo, F. F. Diversidade Florística nas Cangas do Quadrilátero Ferrífero. Na Raiz (2012).

Jacobi, C. M., Carmo, F. F. do, Vincent, R. C. & Stehmann, J. R. Plant communities on ironstone outcrops: a diverse and endangered Brazilian ecosystem. Biodivers Conserv 16, 2185–2200 (2007).

Louise A. Rollins-Smith, Laura K. Reinert, Chadrick J. O'Leary, Laura E. Houston, Douglas C. Woodhams, Antimicrobial Peptide Defenses in Amphibian Skin, Integrative and Comparative Biology , Volume 45, Issue 1, January 2005, 137–142p, (2005).

Markert, Bernd A., Anton M. Breure, and Harald G. Zechmeister, eds. Bioindicators and biomonitors. Elsevier, (2003).

Masindi, Vhahangwele, and Khathutshelo L. Muedi. "Environmental contamination by heavy metals." Heavy metals 10: 115-132. (2018)

Mcgeoch, M.A. The selection, testing and application of terrestrial insects as bioindicators. Biological Reviews, 73, 181- 201. . (1998).

Nascimento, A. C.; Fontes, W.; Sebben, A.& CASTRO, M. S. Antimicrobial peptides from anurans skin secretions. Protein Pept. Lett. 10 , 227-238. (2003).

Nascimento, L. P. D. et al. Avaliação geoquímica de metais em sistemas fluviais afetados por atividades antrópicas no Quadrilátero Ferrífero. Engenharia Sanitaria e Ambiental, v. 23, p. 767-778, (2018).

Navarro, Claudio A; Von Bernath, Diego; Jerez, Carlos A. Heavy Metal Resistance Strategies of Acidophilic Bacteria and Their Acquisition: Importance for Biomining and Bioremediation. Biol. Res., Santiago, v. 46, n. 4, p. 363-371, (2013).

Ndiaye, E., Sandeno, J., McGrath, D., & Dick, R. Integrative biological indicators for detecting change in soil quality. American Journal of Alternative Agriculture, 15(1), 26-36. doi:10.1017/S0889189300008432.(2000).

Nies DH. Heavy-metal resistant bacteria as extremophiles: molecular physiology and biotechnological use of Ralstonia sp. CH34. Extremophiles. (2000).

O’Toole, G.A., Kapplan, H.B., Kolter, R: Biofilm formation as microbial development. Annual Review of Microbiology 54: 49–79. (2000).

Ron, Eliora Z. "Bacterial stress response." The prokaryotes : 1012-1027. (2006).

Salle, A. J. Effect of environment upon bacteria. Fundamental pronciples of bacteriology. 5 ED. New York, Toronto, London: Mcgraw-Hill Book Company, inC., P. 232-267. (1961).

Silva, Fernando Reis. A paisagem do Quadrilátero Ferrífero, MG: potencial para o uso turístico da sua geologia e geomorfologia. 144p. (2007).

Toledo, L. F. Anfíbios como Bioindicadores. In: Neumann-Leitão, S. & El-Dier, S. (Orgs.) Bioindicadores da Qualidade Ambiental. Recife: Instituto Brasileiro PróCidadania. Pp. 196-208. (2009).

Wang S, Zhao X. On potential of biological treatment for arsenic contaminated soils and groundwater. Journal Environmental Manage. Jun;90(8):2367-76, (2009).

Zasloff, M. Antimicrobial peptides of multicellular organisms. Nature 415, 389-395. (2002).