Host-microbiota associations: Characterization of the bacterial diversity in Amphibians across Species, Life Stages and Habitats.

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Nota prévia

Na elaboração desta tese, e nos termos do número 2 do Artigo 4o do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do D.L. 74/2006, de 24 de Março, com a nova redação introduzida pelo D.L. 230/2009, de 14 de Setembro, foi efetuado o aproveitamento total de um conjunto coerente de trabalhos de investigação, um dos quais está actualmente em avaliação por pares e restantes serão em breve submidos a revistas internacionais indexadas e com arbitragem científica, os quais integram os capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua conceção, na obtenção e análise de dados, e discussão de resultados, bem como na elaboração da sua forma aqui apresentada.

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Resumo

As associações simbióticas entre micro-organismos e organismos eucariotas multicelulares são comuns na natureza e têm papéis fundamentais em termos fisiológicos e ecológicos para o sucesso do hospedeiro. O microbioma é influenciado por diversos fatores intrínsicos e extrínsicos ao hospedeiro. A investigação em microbiomas de anfíbios tem vindo a crescer exponencialmente nos últimos anos e tem sido especialmente direcionada para o estudo da comunidade microbiana da pele e, em menor grau, para a comunidade microbiana do intestino. Ambas são extremamente importantes para o anfíbio, afetando o seu metabolismo, crescimento, imunidade e adaptação. Ainda assim, poucos são os estudos que tenham caracterizado a comunidade da pele e do intestino no mesmo indivíduo.

Esta tese tem como objectivos a caracterização da diversidade de bactérias, em anfíbios, associadas à pele e ao intestino, em diferentes espécies de Portugal e Madagascar; tendo em conta a influência de vários fatores ambientais aos quais os anfíbios estão expostos. Os efeitos de fatores ambientais abióticos, nomeadamente o local e tipo de habitat, origem da água e pH, e fatores bióticos incluindo a diversidade bacteriana da água, foram avaliados tanto em populações naturais como através de experiências laboratoriais. A influência de fatores associados ao hospedeiro, tal como a espécie, se é nativa ou invasora, estádio de deesenvolvimento e sexo, foram também analisados.

No Capítulo II foi caracterizado o microbioma da pele de indivíduos adultos de duas espécies nativas de Madagascar, em três locais com diferente influência antropogénica (desde locais sub-pristinos até locais mais modificados). Ao mesmo tempo, avaliamos a influência do pH da água no microbioma da pele, e observamos que houve um efeito significativo na diversidade bacteriana e no número de taxa com propriedades antifúngicas indicando que este fator poderá ter efeitos indiretos na capacidade imunológica do hospedeiro.

No Capítulo III, foi caracterizado o microbioma do sapo comum asiático (*Duttaphrynus melanostictus*), espécie nativa do sudeste Asiático e invasora recente em Madagascar. Avaliamos o microbioma da pele e intestino ao longo da sua zona de expansão e observamos que não há diferenças entre indivíduos ou locais, mas o microbioma da pele da espécie invasora revelou-se significativamente diferente, e mais rico, do que o microbioma da pela da espécie de rã nativa que ocorre nos mesmos locais.

No Capítulo IV, investiguei as alterações no microbioma da pele e do intestine ao longo do processo de metamorfose em três espécies de anfíbios (dois anuros e um urodelo), amostrados na mesma charca temporária no norte de Portugal. A espécie do hospedeiro revelou ser um efeito significativo na diversidade de bactérias da pele, com os anuros a exibirem comunidades mais similares entre si do que o urodelo. Os estádios de desenvolvimento indicaram que o recém metamorfo, acabado de emergir do meio aquático, tem a comunidade mais distinta, evidenciando o contraste entre os estádios aquáticos e terrestres. A comunidade bacteriana do intestino revelou menor variabilidade entre espécie de hospedeiro e estádio de desenvolvimento, sendo que as diferenças observadas foram maioritariamente em níveis de abundância relativa e estrutura da comunidade. O urodelo revelou ter uma comunidade de simbiontes mais similar com a do meio aquático, em comparação com os dois anuros, sugerindo que cada hospedeiro deverá ter diferentes processos de filtragem de bactérias colonizadoras a partir do ambiente.

Finalmente, no Capítulo V, usei um ensaio laboratorial controlado para simular e analisar o efeito do contacto com um ambiente diferente no microbioma da pele e intestino. Para isso, expus girinos de duas espécies à sua água nativa e a água do local da segunda espécie. Foi observado que a identidade do hospedeiro tem uma grande influência na comunidade bacteriana da pele e a origem da água constitui um fator secundário, também significativo. Quanto à alteração ao longo da experiência, foi observado queas comunidades bacterianas da pele e intestino de diferentes hospedeiros exibiram padrões de resposta distintos quando os girinos foram expostos a água não-nativa, sugerindo que a comunidade bacteriana original do hospedeiro e a identidade do hospedeiro influenciam a resposta da comunidade bacteriana quando sujeita a alterações de efeitos ambientais.

Esta tese contribui para o aumento do conhecimento relativo ao microbioma de anfíbios, incluindo uma espécie invasora. Comparando os padrões de duas comunidades diferentes (pele e intestino), expostas a diversas condições ambientais, pude explorar as respostas específicas e de desenvolvimento aos mesmos fatores abióticos e bióticos. Finalmente, através do uso de um ensaio laboratorial, pude individualizar os efeitos de cada fator, enquanto que a caracterização de comunidades bacterianas de populações naturais permitiu uma melhor compreensão de como as comunidades simbióticas de vários anfíbios estão a mudar na natureza.

Palavras-chave: Anfíbios, Bactérias, Simbiontes, 16S rRNA, Pele, Intestino, Espécie, estádios de vida, Habitat, 16S rRNA.

Abstract

Symbiotic associations of microorganisms with multicellular eukaryote are widespread in nature and have fundamental physiological and ecological roles for the host fitness. The microbiome is affected by several host-extrinsic and intrinsic factors. Research on amphibian microbiome has grown exponentially in recent years and has been especially focused on the study of skin-associated communities and to a lesser extent of gut-associated communities. Both are of extremely importance for the amphibian host affecting its metabolism, growth, immunity and adaptation. Still, few studies have addressed skin and gut communities in the same individual. This thesis aimed to characterize the amphibian bacterial diversity associated to the skin and the gut in different host species from Portugal and Madagascar, while taking into account the influence of several distinct factors to which amphibians are exposed. The effects of environmental extrinsic abiotic factors, namely site and habitat type, water source and water pH and biotic factors including water bacterial pool were assessed including using both natural populations and laboratorial experiences. The influence of host-intrinsic factors such as host species, native-invasive status, developmental stage and sex were also analyzed for some of the target amphibian species.

In Chapter II, we performed a characterization of the skin microbiome of adults of two native Malagasy amphibian species across three sites characterized by distinct anthropogenic influence (from sub pristine to more anthropized areas). At the same time, we evaluated the influence of water pH on the skin microbiome and found that it significantly affected bacterial diversity and the number of taxa with antifungal properties indicating that this abiotic factor may have indirect effects on the immune capacity of the host.

In Chapter III, we characterized the microbiome of the Asian common (*Duttaphrynus melanostictus*) toad, a species which is native from south East Asia and that it is invasive in Madagascar. We studied the skin and gut microbiota across the expansion range of the species and found no differences between individuals from different sexes or sites, but found that the skin microbiome of the invasive species was significantly different and more diverse from the skin microbiome of a co-occurring native frog species.

In Chapter IV, we investigated the temporal alteration of skin and gut microbiome across the metamorphosis process in three amphibian species (two anurans and one urodele) that were sampled syntopically in a temporal pond in northern Portugal. Host species had a significant effect on skin bacteria diversity with anurans exhibiting similar communities that the urodele while development stages indicate that the neometarmoph, recently emerged from the water has the most distinctive community highlighting the contrast between aquatic and terrestrial stages. The gut bacterial community was less influenced by host species and developmental stages mostly exhibiting differences in terms of relative abundance levels and community structure. The urodele carried a symbiotic community more similar to the water environment then the other two amphibians suggesting different host filtering process in the skin and gut of each host.

Finally, in Chapter V, we performed a laboratorial experiment to simulate and analyze the effect of the contact with a different environment on the skin- and gut-associated microbiome. We used tadpoles from two species and exposed them to their native water and to their reciprocal translocated water sources. We found that host identity has a strong influence on the skin bacterial community with water source being the second significant factor. Moreover, it was observed that in controlled conditions, that skin and gut bacterial communities from different hosts exhibit different response patterns.

This thesis contributes to increase the knowledge of amphibian's microbiome including an invasive species. By comparing the patterns using two different communities (skin and the gut), under several environmental conditions, I could explore their specific responses to the same biotic or abiotic factors. Finally, while using a laboratory experiment, I could individualize the effects of each factor, while characterizing the bacterial communities of natural populations I could get a better understanding of how symbiotic communities of several amphibians are changing in the wild.

Keywords: Amphibians, Bacteria, Symbionts, 16S rRNA, Skin, Gut, Host Species, Life stage, Habitat, 16S rRNA.

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***Bd* -** *Batrachochytrium dendrobatidis*, a pathogenic chytrid fungi

***Bsal* -** *Batrachochytrium salamandrivorans*, a pathogenic chytrid fungi

EMP – Earth Microbiome Project

HMP – Human Microbiome Project

LEfSe - Linear discriminant analysis Effect Size; bioinformatic software package to identified features (e.g., organisms, genes, operational taxonomic units) that most likely explain differences between classes.

NMDS – Non-metric multi-dimensional scaling ordination analysis

PICRUSt - Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; bioinformatic software package to predict metagenome functional content from the 16S rRNA marker gene.

sOTUs - sub-operational taxonomic units

List of Definitions

Alpha diversity (α-diversity) - describes diversity of species or other taxa within a sample.

Beta diversity (β-diversity) - describes differences in diversity among groups of samples.

Chytridiomycosis – disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* or *B. salamandrivorans*

Dysbiosis – microbial imbalance in a community

Holobiome - collective genomes of both host and symbionts

Microbiome – group of microbes (symbiotic, commensal or pathogenic) and their collective genome living inside or on the host body or other environment

Microbiota - group of microbes (symbiotic, commensal or pathogenic) in a specific environment

Phylosymbiosis – when microbial community relationships recapitulate the phylogeny of their host'

CHAPTER I

General introduction

1.1. Microbes and Microbiome

1.1.1. Defining Microbes, Microbiota and Microbiome

Microbes or microorganisms includes Prokaryotes (Bacteria and Archaea) and Eukaryotes (e.g., Protozoa, Algae and Fungi) and can be found in high abundance in every environment (Pepper et al., 2009). Bacteria, in particular, are among the most common and widespread, which diversity is unfathomable vast and are essential components in every ecosystems (DeLong & Pace, 2001; Fuhrman, 2009). They possess a vast array of chemical and biological properties (Pepper et al., 2009) and are often organized in dense multispecies communities with complex interactions as free-living individuals or symbiotic assemblages. In the last few decades, the study of microbial diversity associated with animal and plant hosts – Microbiota or Microbiome, has increased exponentially (Reese & Dunn, 2018; Ross et al., 2019). Reviewed by Joshua Lederberg (2001), these terms have been used since much earlier. Prescott (2017) highlights that the term Microbiota was already in use in the 1960s in laboratorial experiments with germ-free animals to study the inoculation with specific bacterial communities to protect the hosts from infections by other microorganisms (Lane-petter, 1962); on the other hand, the term Microbiome was often used to refer to a community within specific ecological settings, the definition currently used in microbiology research. Nowadays, there is the need to find a consensus for a common definition. The currently most used definition for the term Microbiome is described within an ecological context and includes all the microbes (symbiotic, commensal or pathogenic) and their collective genome including all groups (e.g. bacteria, archaea, fungi, protozoans and viruses) living inside or on the host body or other environment, while Microbiota is defined as the assemblage of all the living microorganisms members in a specific environment, although the two terms are often used interchangeably (Berg et al., 2020; Marchesi & Ravel, 2015).

1.1.2. A brief history of the study of microbial communities

The study of microbes started from the necessity to understand human pathogens but quickly expanded to a wider environmental context, to include the characterization of microbial communities in model organisms, natural habitats and environmental layers (e.g. soil, water and air), among others (Pepper et al., 2009). The creation of the microscope allowed the discovery of this organisms named “animalcules” by Anthony van Leeuwenhoek in 1670 (Berg et al., 2020). In the past, microbial research was mostly conducted using culturing methods and light microscopy, and species identification was based on their morphology, biochemistry and ecology (Grigorova & Norris, 1990). Characterizing community’s richness would be performed by counting microorganisms, and their ecology would be investigated through exposing cultures to different growth media (Grigorova & Norris, 1990; Kemp et al., 1993). However, only a small portion of microbe diversity is culturable, and the early diagnostic characters were quite limited. The advent of DNA sequencing and molecular biology allowed classifying bacteria based on DNA similarity using 16S ribosomal RNA (rRNA), and has revolutionized bacterial systematics (Medini et al., 2008; Sanger et al., 1977), but the vast majority of microbial diversity would however remain unexplored. In the last 20 years, new sampling methods combined with an exponential increase in data generation and the decrease in sequencing costs have revolutionized microbial research field (National Research Council, 2007). The “Metagenomics Era” target the whole environmental community diversity instead of focusing on singular organisms and allowed to reveal a great amount of previously unknown bacterial taxa (National Research Council, 2007). Although culturing methods remain an important component, it is currently common to collect DNA samples from tissue samples (invasive methods) or by swabbing the target surface (non-invasive methods) and to sequence targeted DNA regions or even whole bacterial genomes in a short time and for a relatively reduced cost (Metzker, 2010). These sampling and laboratorial advancements have been accompanied by increasing development of computational power and analytical methods, with new software and pipelines emerging frequently. All this is facilitating the rapid development of this field of research, opening new possibilities and expanding its frontiers.

1.1.3. Recent advances and contemporary scenario

The study of microbiomes currently operates at a global level, both in terms of studied subjects and research teams. Among the most know broad-scale projects are Human Microbiome Project (HMP; https://hmpdacc.org/) and the Earth Microbiome Project (EMP; https://press.igsb.anl.gov/earthmicrobiome/). The HMP started in 2008 and involves a global sampling across different geographic areas, gender and age, genetic background, health and economic status, among many other variables, bringing major advances in our understanding of the human-associated microbes and their role in host's health. The EMP that started in 2010 aims to apply standardized protocols to identify the microbial communities living in water, soil, sediment, plants, biofilms and animal hosts, in order to characterize the broad patterns of global microbial diversity (Thompson et al., 2017).

Currently, the most common method used to characterize microbial communities is amplicon-based sequencing, although other recent methods are rapidly increasing (Deurenberg et al., 2017). Typically, one or a limited set of gene fragments are targeted and sequenced from the entire microbial community of a given sample. The different target genes depend on the microbe group: the 16S ribosomal RNA (16S rRNA) gene is preferred for bacteria and archaea (Liu et al., 2012), while the 18S rRNA, the 28S rRNA and ITS1-2 are generally chose to characterize fungi and protozoans (Schoch et al., 2012). For the 16S marker, one out of the nine hypervariable regions (named as V1 to V9) can be chosen depending on its taxonomic resolution (Bukin et al., 2019). The V4 region obtained using the 515F (GTGCCAGCMGCCGCGGTAA) - 806R (GGACTACHVGGGTWTCTAAT) (10µM) primers pair (Caporaso, et al., 2010) is the one recommended by the Earth Microbiome Project in the last years. Sequence data is normally stored in massive open access repositories (e.g., NCBI (www.ncbi.nlm.nih.gov), or the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/browser/home)), which facilitates building upon previous research anywhere in the world. Currently, there are three major reference databases that are used to assign taxonomy: Greengenes (DeSantis et al., 2006), Ribossomal Database Project (RDP) (Cole et al., 2014) and SILVA rRNA Database Project (SILVA) (Quast et al., 2013). However, databases differ in sequence content, quality checks and meta-data, which hinder a streamlined identification of microbiota taxa and extend the time of processing of microbiome raw data, especially for non-model hosts. Regardless of the used reference database, the proportion of referenced taxa is still minuscule comparing with the expected bacterial diversity (Louca et al., 2019). These reference databases are

mostly populated by bacteria taxa found associated with human hosts, model organisms and soil communities and therefore, several OTUs identified in non-model organism cannot find a reliable match. On the other hand, the recent development of open-access software and the availability of several analytical pipelines enabled the establishment of rapid quality filtering and facilitate data comparison, but the extremely fast expansion of microbial studies have been accompanied by several new challenges (Berg et al., 2020).

1.2. Host-microbe symbiosis

In a perspective issue release in 2013, McFall-Ngai and colleagues stressed that it is crucial to consider that animals live within a bacterial world (Mcfall-Ngai et al., 2013), highlighting that many bacteria have been evolving for billions of years in symbiotic relationships with eukaryote groups, making up a significant component of the host, and these symbiotic relationships are nearly ubiquitous – the “Holobiont” concept (Bordenstein & Theis, 2015).

The “Hologenome” is a term that refers to the collective genomes of both host and symbionts (Bordenstein & Theis, 2015), while the “Bacteriome” refers to the bacteria community and their genome. Other symbiotics communities that have been receiving increasing attention are the microeukaryotes (with the “Eukaryome” referring to the eukaryote pool and their genome). Among the eukaryotes, the fungal community (“Mycobiome”) and the microeukaryote parasites are the ones that have received more attention, and therefore more data on these groups are currently available (Campo et al., 2019). All symbionts, either bacteria or microeukaryotes, have complex interactions among them and within the host, and can be beneficial, commensal or pathogenic (Bernardo-Cravo et al., 2020). A great proportion of the data on host-microbiome interactions comes from the study of human microbiome, a direct consequence of the HMP, with microbiome studies on model species (e.g., mouse, *drosophila*) and industrially produced organisms (e.g., aquaculture fish) being the next major source of information. Microbe-free eukaryotes exposed to known microorganisms (gnotobiotic organisms, from Greek roots gnostos "known" and bios "life", i.e. all species components of the holobiont are known) are particularly interesting to study host-microbe and microbe-microbe interactions, especially related with health-disease characterization studies (Gordon & Pesti, 1971). The expansion of this research to non-model organisms and natural ecosystems brought the interest of host-microbiome interactions beyond human health and animal and plant farming. For example, researchers

are investigating the role of microbes in the stability and ecosystem health (e.g. aquatic environments, forest, agriculture areas), assessing microbial effects on host adaptation and fitness and exploring ways to include symbionts in conservation plans of endangered species (Antwis et al., 2019; Bernardo-Cravo et al., 2020; West et al., 2019)(Fig. 1); on the other side, they are studying how the ecology of the host is influencing its microbiome [e.g., migration, hibernation, diet patterns, socialization status (Ambrosini et al., 2019; Carey et al., 2012; Greene et al., 2020), how human activities affect the microbial pool in the ecosystem (Mallin et al., 2000), or how evolution may influence host-microbiome associations (Loo et al., 2019; Youngblut et al., 2018)], and remain crucial to understand how the symbiotic communities are shaped and what are the major factors influencing this host-symbiont relationship (Antwis et al., 2017).

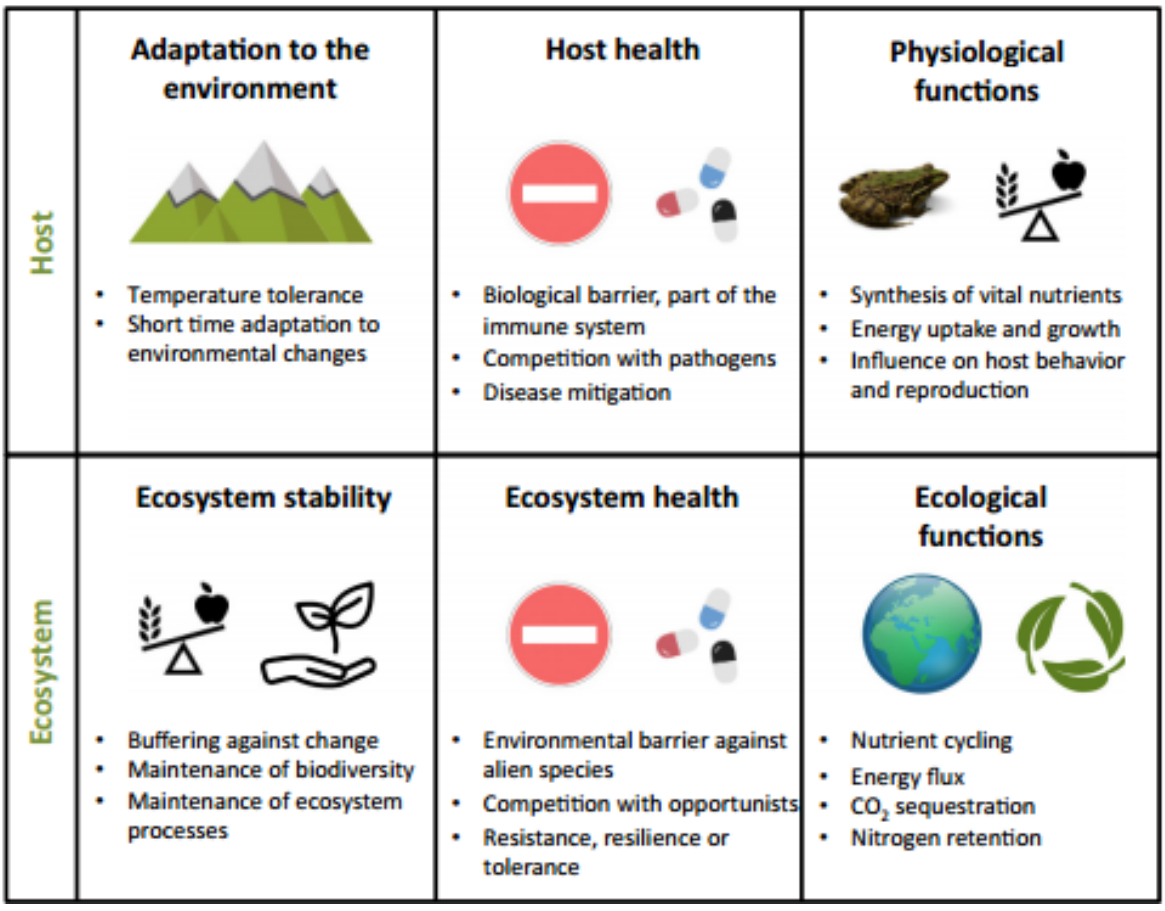

Figure 1. 1 The important functions of microbiome to hosts and ecosystems. Figure retrieved from Bernardo-Cravo et al. (2020).

1.2.1. Host extrinsic/exogenous factors (Abiotic and Biotic)

Symbiotic bacterial communities can be influenced by single or combined biotic and abiotic factors (Jiménez & Sommer, 2017; Reese & Dunn, 2018)(Fig. 2). Abiotic factors can be climatic (e.g. temperature, humidity), geographic (e.g. site, altitude, latitude) chemical (such as pH, nutrients, turbidity and pollutants), among others (Griffiths et al., 2019; Hughey et al., 2017; Krynak et al., 2015; Sylvain et al., 2016). These can influence bacterial communities by changing their taxonomic composition, abundance or functional diversity (e.g. (Krynak et al., 2015; Kueneman et al., 2019) or altering host susceptibility to pathogens (Hess et al., 2015). These alterations have been observed in environmental samples (such as soils or waterbodies; Andersson & Nilsson 2001, Alkorta et al. 2017, Li et al. 2018, Zhang et al. 2018) and in animal or plant hosts (Mina et al., 2020; Sylvain et al., 2016; Tajima et al., 2007). Among climate variables, temperature is one of the most investigated parameters and showed to have a major effect on microbiota richness and structure in animal gut (Sepulveda & Moeller, 2020). Habitat alterations by human pressure such as the modification of forest areas into agriculture fields can also have impacting effects on both environmental of symbiotic microbes. For example, Chen and colleagues (Chen et al., 2018) found that small streams within agricultural lands have richer diversity of aquatic bacterial communities than larger aquatic bodies do, suggesting that in similar conditions, geomorphology can also represent an important environmental variable. In another study, two populations (lake versus pond) of the Cyprinidae fish species *Tinca tinca* exhibited differences both in terms of richness, diversity and composition in the gut bacteria, with populations from the lake harboring richer communities (Dulski et al., 2020). On the other hand, in some groups, the responses to habitat modification are greatly dependent of the host species (Tiede et al., 2017).

Differences among habitats are intrinsically related with environmental parameters such as type of vegetation, soil and water uses and management, pH, nutrients, oxygen, pollutants, among many others. Besides the well-known effects of habitat alteration on reducing native land, altering ecosystems and limiting natural resources for wild populations (e.g., vegetation, water, land, hiding spots), these have a significant impact also on environmental microbes and symbiotic communities from several animals and plants (Chang et al., 2016; Chen et al., 2018; Huang et al., 2018). In a threatened species of monkey from Tanzania, individuals occurring in human-impacted forests exhibited a poorer gut bacterial community and increased metabolic needs to digest xenobiotics, due to the alteration of available plant

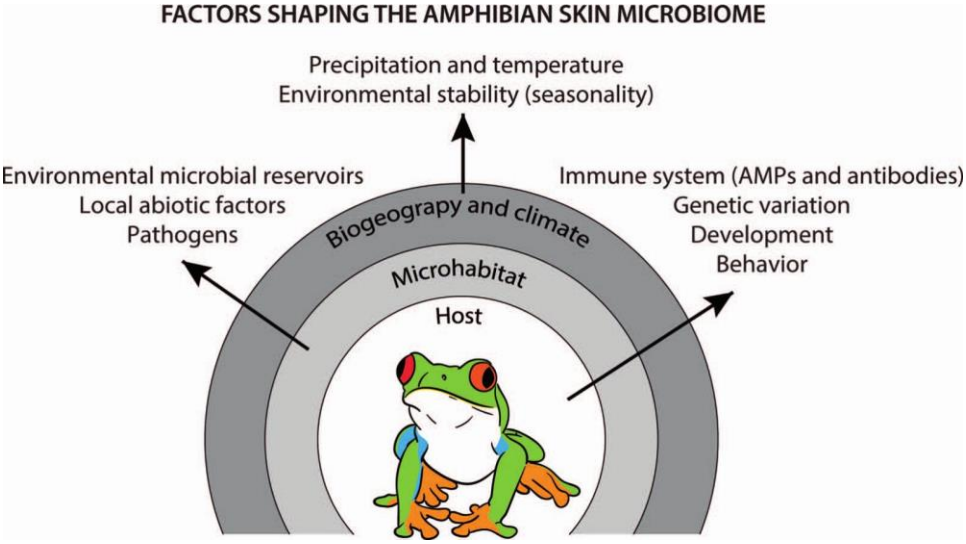

Figure 1. 2 Biotic and abiotic factors shaping the amphibian skin microbiome. Figure retrieved from Rebollar et al. (2020).

diversity (Barelli et al., 2015). On the other hand, the exposure to soil and water contaminants, such as herbicides and insecticides, greatly reduce bacterial community diversity in aquatic systems (Muturi et al., 2017), as well as the bacterial diversity associated to an animal host (e.g. in gut of adult amphibians; Knutie et al. 2018). These alterations can impair the gut symbiotic community and functionality (dysbiosis) and can have severe implications in population success. Other factors, such as drastic alterations in water pH levels was found to cause disruption of the skin microbiota of fishes and in turn facilitate pathogens' invasion (Sylvain et al., 2016).

Biotic factors impacting host-microbiome associations include environmental microbial reservoirs, pathogens, interactions among hosts (e.g., competition, predation, parasitism, co-occurrence, invasive species), food resources, space availability, among others (Jiménez & Sommer, 2017; Rebollar et al., 2020; Sandionigi et al., 2015). Several studies found that a portion of microbiota is often shared among co-occurring species and with the environmental layers (e.g., water, soil, plants) (Mcfrederick et al., 2017). The shared taxa between hosts may result from the direct exposure to a common surrounding environment, but can also be due to the contact between hosts and horizontal transfer of specific microbial taxa. For example, in a study with bats, the authors compared individuals from a species A that where sharing the same space or not with a species B. Individuals from species A exhibited more similar skin microbiome to the co-housing species B than with individuals of its own species that where inhabiting in a separate habitat, indicating a convergence of their

symbiotic bacterial communities that could partly result from the transfer of microbes between the two species and the exposition to the same environmental microbial pool (Lemieux-Labonté et al., 2016). Diet type and prey availability can also alter the gut communities, with different groups of bacteria playing a wide range of physiological roles including aiding to the digestion of different substrates and promoting nutrient uptake and detoxification (Grond et al., 2018). In fishes from different trophic levels (carnivorous versus herbivorous) the symbiotic communities were highly distinct in terms of diversity and composition and these changes were related to metabolic roles (e.g. cellulose degradation and protease production; Liu et al. 2016). Few studies tried to evaluate the effects of different diets on gut microbiome in non-model organisms. Among these, in a recent study, the authors performed a laboratory feeding experiment with beetles, and observed that gut bacterial richness increased while increasing the number of different prey items (Tiede et al., 2017).

1.2.2. Host intrinsic/endogenous factors

Although environmental and habitat features have a great influence on microbial communities, factors related with the individual host are also extremely relevant (Fig. 2). These include genetic background, age, sex, ecology and behavior, physiology and health status, among others (Jiménez & Sommer, 2017). The host species, age and sex are among the most studied factors in both model organisms and natural populations. Age/stage-dependent microbial changes are very common in symbiotic communities, and have been observed for instance in gut community of birds, where nestlings exhibited bacterial assemblages that differ in taxa composition and abundance from adults (Kohl et al., 2019) or in the skin symbionts from tropical fishes (Xavier et al., 2020). In *Drosophila*, a well-known model organism including in microbiome research, gut bacterial abundances greatly differ between sexes and across developmental stages (Han et al., 2017). Dramatic changes in the microbiome of aged *Drosophila* individuals were linked to a rapid decline in gut functions and host health prior to death (Clark et al., 2015). In humans, the evolution of the gut bacteria in the aging process has been vastly investigated and revealed that the phylogenetic composition greatly changes in the first three years after birth, stabilizing afterwards until adulthood while the reduced gut diversity in older adults has been linked to higher susceptibility to disease (Cuesta-Zuluaga et al., 2019; Nagpal et al., 2018). Age effects were also detected in the human skin microbiome, with adults exhibiting greater diversity than

younger and older counterparts (Ying et al., 2015). However, skin properties such as moisture level, can also greatly influence the skin microbiota abundance levels of specific taxa (Ying et al., 2015). The influence of sex in determining bacterial diversity is found in several groups of organisms including amphibians (Griffiths et al., 2018) and in small mammals (Lavrinienko et al., 2018).

1.2.3. Phylosymbiosis and Coevolution

Interactions between different species can result in a reciprocal effect on their evolutionary history from the selective pressure imposed on each other, and it is defined as coevolution. Examples include predator-prey, parasite and host' immune system or mutualist relationships like pollinators and entomophilous or ornithophilous flowers (Brown & Vincent, 1992; Kiester et al., 2013; May & Anderson, 1990). Phylosymbiosis occurs when the host phylogeny is reflected on the similarity dendrogram of their microbiota (Brooks et al., 2017), although this does not necessarily imply coevolution with each microbe. Across wild animal groups, such as primates and birds, host species seems to be a strong factor affecting gut-associated symbiotic communities (Amato et al., 2017; Hird et al., 2015). These observations had gathered curiosity as whether this scenario would be common in other groups. Indeed, this pattern was observed in the gut microbiota of other mammals (Groussin et al., 2017; Youngblut et al., 2018), in the skin-associated microbiota of coral reef fishes (Chiarello et al., 2018), and in sponges (Easson & Thacker, 2014), although in most cases host phylogeny also reflected ecological traits, geographic distribution and habitat parameters (Bird et al., 2018; Chiarello et al., 2018; Sullam et al., 2012), and it is therefore difficult to disentangle the effect of one or the other factor. The gut environment seems to exhibit particularly stronger signs of phylosymbiosis than other bacterial communities and it may have a significant impact on host fitness and adaptation capacity (Moeller & Sanders, 2020). However, several studies accounting for host phylogeny and host ecological traits or habitat, found that the ecological factors tend to have stronger correlation to microbial composition than host phylogeny (Bletz et al., 2017; Loo et al., 2019).

1.2.4. Physiology and Immunity

Symbiotic bacteria can aid the host in several physiological functions, among the most studied being digestion and immune response (Knutie et al., 2018; Knutie et al., 2017; Koch & Schmid-hempel, 2011; Moeller & Sanders, 2020). This microbiota roles have been particularly explored for skin and gut bacterial communities across different hosts (Ross et al., 2019), especially in humans and model organisms (Clemente et al.,, 2012), but also in aquaculture systems (Perry et al., 2020). Due to the high incidence of infectious diseases in aquaculture systems, it is expected that host skin bacteria composition may be affected, and in turn also the host susceptibility to pathogens, as it has been observed in fishes (Rosado et al., 2019). Skin microbiota can deter pathogens through competition, by producing anti-bacterial molecules, or stimulating host immune response (Grice & Segre, 2011; Thaiss et al., 2016). In amphibians, it has been found that early-life disruption of the skin microbiota may affect the disease resistance at later adult stages, highlighting important long-term effects on the individual' health (Knutie et al., 2017). Besides competing with other microbes, symbiotic gut bacteria act in combination with intestinal epithelial cells and mucosal structure to ensure proper digestion (Colombo et al., 2015; Iacob & Iacob, 2019), with an unbalance in species diversity and abundance potentially leading to gut dysbiosis and to a poorer healthy host (Smith et al., 2017).

1.3. Amphibians

1.3.1. Main characteristics

Amphibians are a very diverse group of organisms with more than 8,000 described taxa divided into three orders: Anura which include frogs and toads (around 88% species); Caudata or Urodele that include salamanders and newts (9% species); and Gymnophiona, the limbless caecilians (3% species) (Duellman & Trueb, 1986). The three groups have different geographic distributions (Wake & Koo 2018): anurans occur in all the continents with exception of Antarctica and are especially diverse and abundant in the tropics; urodeles are distributed mostly in the Northern hemisphere; while the gymnophiones have the most restricted distribution and occur only in the tropics. Amphibians can be found in almost all type of habitats from rainforests to deserts, and from the tropics to the Arctic circle (Hedges & Kumar, 2009; Wells, 2007). Each group has very distinct morphologic and ecologic features and very different life histories (Wake & Koo 2018). They are frequently very

dependent on aquatic habitats, which is well reflected by their morphology and physiology. Within anurans, frogs are often associated with aquatic environments also at the adult stage while toads are more robust and tend to be more independent from water (although remain linked to this environment for their reproduction); neither adult anuran nor caudata have gills, although there are some notable exceptions in salamanders (Wake & Koo, 2018). Caecilians are the most distinct group exhibiting a worm-like elongated body, lacking limbs and usually tail. The majority of the species begin their life cycle in aquatic environment as shell-less eggs, then hatch into tadpoles (anuran), larvae (urodele and caecilians), and then metamorphose and often move to the terrestrial habitat (Duellman & Trueb, 1986). Metamorphosis entails drastic morphological and physiological changes. Anurans typically go from an aquatic herbivorous/detritivores tadpole stage to a four-legged carnivorous adult, while urodeles maintain a carnivorous diet throughout their life cycle. Example of notable variations to this pattern includes direct development from eggs (Townsend & Stewart, 2012), viviparity (Buckley, 2012) and neoteny (Reilly, 1987). The amphibian' skin has no hairs, scales or feathers and has a high level of vascularization and permeability, allowing gas exchanges, osmoregulation and thermoregulation (Wells, 2007). Depending on the group, the skin can be smoother (e.g., frogs) or more rugous (e.g., toads) and can presents glands that produce secretions with physiological or defense functions (Clarke, 1997).

1.3.2. The amphibians in a worldwide biodiversity crisis

Amphibians are among the most threatened groups of vertebrates worldwide (Fig. 3), with nearly half of the species being highly vulnerable or declining (Bishop et al., 2012; IPBES, 2019; Stuart et al., 2004). Their unique life history traits and dependence on both terrestrial and freshwater ecosystems (with the latter being the most threatened systems due to overexploitation and pollution), expose them to a multitude of threats (Duellman & Trueb, 1986). The amphibian's naked and permeable skin makes them heavily exposed and susceptible to environmental stressors such as temperature and humidity fluctuations as well as to water contaminants. This susceptibility to microhabitat characteristics is especially linked to species traits and seems to be phylogenetically conserved in amphibians leading to the loss of species diversity and to a more homogeneous communities where only the most resilient species survive (Nowakowski et al., 2018). Habitat specialists amphibian species (e.g., climate restrictions or narrow distributions) are especially affected (Pyron, 2018).

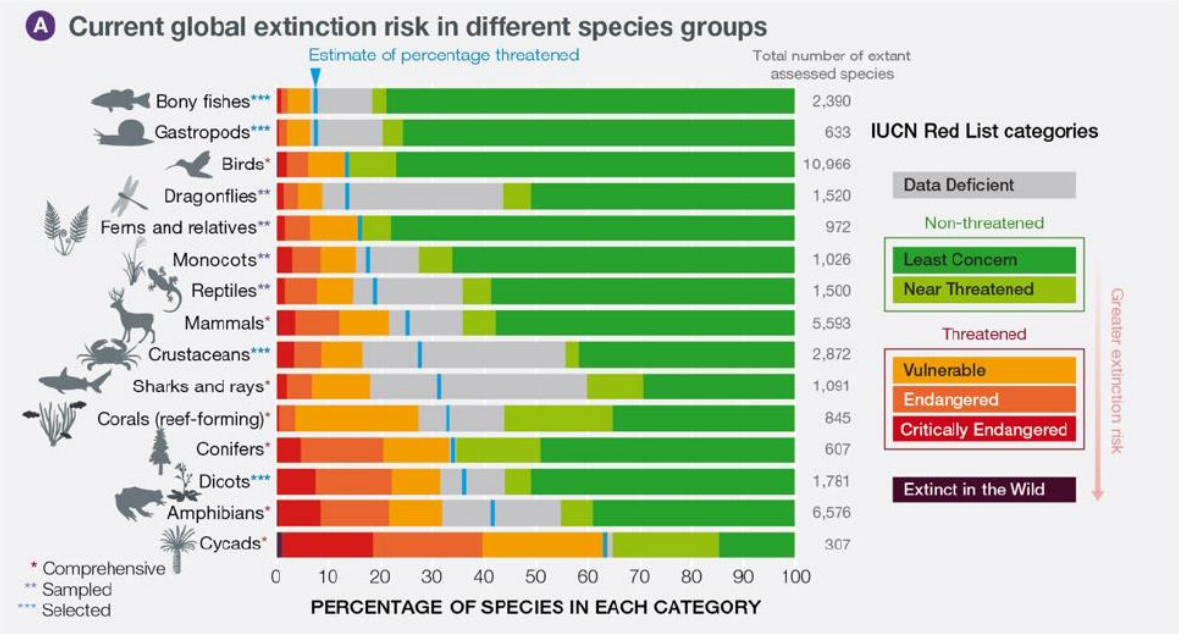

Figure 1. 3 Figure retrieved from IPBES (2019) with graph showing the percentage of species in each category from the IUCN Red List.

Climate change, habitat destruction and fragmentation, pollution, overexploitation, invasive species and emerging infectious diseases are among the leading causes of the worldwide amphibian decline (Bishop et al., 2012; Collins, 2010; Duellman & Trueb, 1986; Hof et al., 2011; Wake & Koo, 2018). Climate change, with temperature rising and humidity and salinity changes in coastal wetlands, typically inhabited by amphibians, is worsening the negative effects of increasing ecosystem pollution (Blaustein et al., 2010; Santos et al., 2013). Habitat destruction and anthropogenization not only reduces available natural space and ecological niches for amphibians but also acts as an important driver of environmental alterations (e.g. low carbon sequestration and temperature increase) (Köhl et al., 2015), with only a handful of species thriving in these human-altered scenarios, often opportunistic and invasive amphibian species (Nowakowski et al., 2018).

Invasive species are a major threat to amphibians and other small vertebrates worldwide (Bishop et al., 2012; Johnson et al., 2011). They can have brutal impacts on ecosystems, by competing for ecological niches and food resources with similar species, by preying or being preyed by native species, or functioning as vectors to pathogens (Bishop et al., 2012; Courant et al., 2017) with impact on islands being typically more severe (Spatz et al., 2017). In amphibians, one of the best documented invasions is the one of the Cane toads (*Rhinella*

marina), native to South and Central America and introduced in Hawaii, Caribbean and Australia, among other places. This toxic toad has caused continuous declines of the native fauna (mostly predators), and while its invasion continue is incessantly posing new challenges to native ecosystems (Shine, 2010). Another textbook example of a successful invasive amphibian species is the American bullfrog *Lithobates catesbeianus*, native to North America and introduced worldwide as a food source (Snow & Witmer, 2010) that has caused severe amphibian population declines (Johnson et al., 2011). Similarly, the African clawed-frog *Xenopus laevis* was introduced in America, Europa and Asia and there are evidences of this species causing reproduction declines in native frogs (Lillo et al., 2011). The reporting of the toxic Asian toad (*Duttaphrynus melanostictus*) in Madagascar has recently catalyzed the attention toward invasion biology (Kull et al., 2014). First reported near the seaport town of Toamasina (eastern coast of Madagascar) in 2014 (Crottini et al., 2014), it is suspected to have arrived in shipping containers around 2010 (Moore et al., 2015; Vences et al., 2017) and is now rapidly expanding its range (Licata et al., 2019, 2020). The species is likely to pose major threats to native fauna including both mammals, reptiles and frogs either by poisoning its predators or by competing with other amphibians for natural resources and reproductive habitats (Andreone et al., 2014; Crottini et al., 2014). Symbionts associated with invasive species can play an important role in the invasion history success of the host and this has been particularly addressed in plants and insects, where it has been found that the microbiome can confer adaptive advantages to the host (Cheng et al., 2018; Coats & Rumpho, 2014).

1.3.3. Amphibian pathogens

The last few decades saw a global rise of infectious diseases, affecting a multitude of aquatic and terrestrial organisms such as amphibians, fishes, corals, bats, among others (Blehert et al., 2009; Fisher et al., 2012; Mera & Bourne, 2018; Toranzo et al., 2005). The rapid emergence and expansion of new pathogens (e.g., bacteria, fungi, viruses) has often been linked to climate change, destruction of the ecosystems and globalization (O'Hanlon et al., 2018). This increase of infectious diseases has been particularly devastating for amphibians, with several reported cases of drastic population declines and numerous extirpations and extinctions (Collins, 2010; Fisher & Garner, 2019; Lips, 2018). Among the most studied pathogens and diseases associated with the worldwide process of amphibian decline are the chytrid fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans* (hereafter *Bd* and

Bsal) that cause chytridiomycosis disease (Fisher & Garner, 2019; A Martel et al., 2014); *Ranavirus* (Price et al., 2014); and the "Red leg syndrome" generally caused by Gram-negative bacteria with a worldwide distribution and infecting both anurans and caudata (Densmore & Green, 2007).

Bd has been receiving significant attention in the last decades due to its dramatic effects including the capacity of infecting anurans, urodeles and caecilians and its occurrence almost everywhere except Antarctica and Papua New Guinea (Bower et al., 2019; Fisher et al., 2009; Olson et al., 2013). Amphibian's unique intake of water and gas across the skin helps maintaining electrolyte homeostasis (Campbell et al., 2013). In adult amphibians, *Bd* damages the outer keratin layer of the skin and inhibits epithelial Na^+ channels, causing the disruption of the skin vital functions leading to electrolyte depletion and osmotic imbalance, and therefore reducing the efficiency of the respiratory and osmoregulatory exchanges (Campbell et al., 2013). *Bd* infection attenuates Na^+ absorption through the skin and in severely affected individuals provokes the systemic depletion of Na^+, K^+ and Cl^- causing a deterioration of cardiac electrical function, leading to the dead of the affected individual (Campbell et al., 2013). In tadpoles, the mouthpart is the only keratinized body structure and therefore the only part being directly affected by *Bd* (Berger et al., 1998). Infected tadpoles were observed to forage less and with lower efficiency than uninfected tadpoles, leading to starvation and often preventing a successful metamorphosis (Venesky et al., 2010). One of the most intriguing aspects of the global *Bd* pandemic is the large spectrum of host susceptibility (Woodhams et al., 2007), with species undergoing mass-mortalities (Gillespie et al., 2015), and others coping with the infection, such as the North American bullfrog *Lithobates catesbeiana*, introduced globally and acting as carrier of the fungus (Borzée et al., 2017). Some of the reasons proposed to explain this variability in susceptibility include habitat variables, climatic parameters (e.g. temperature and seasonality) and host-intrinsic characteristics (e.g. immunological system, skin secretions, skin bacteria; Richmond et al. 2009, Bates et al. 2018). On a more optimistic view, an increasing number of studies have been found that some amphibian species harbor bacterial taxa with antifungal properties that can inhibit *Bd* growth and other fungi (Harris et al., 2006) and that some strains were associated with *Bd*-resistant hosts (Becker & Harris, 2010) opening the way to the application of bacterial taxa as probiotics in amphibians conservation efforts (Bletz et al., 2013). However, although some taxa seems to exhibit these properties, it is still needed a more complete analysis and characterization of symbiotic bacterial taxa that may have

broad-scale spectrum of pathogen inhibition to build effective protective microbiomes (Antwis et al., 2015).

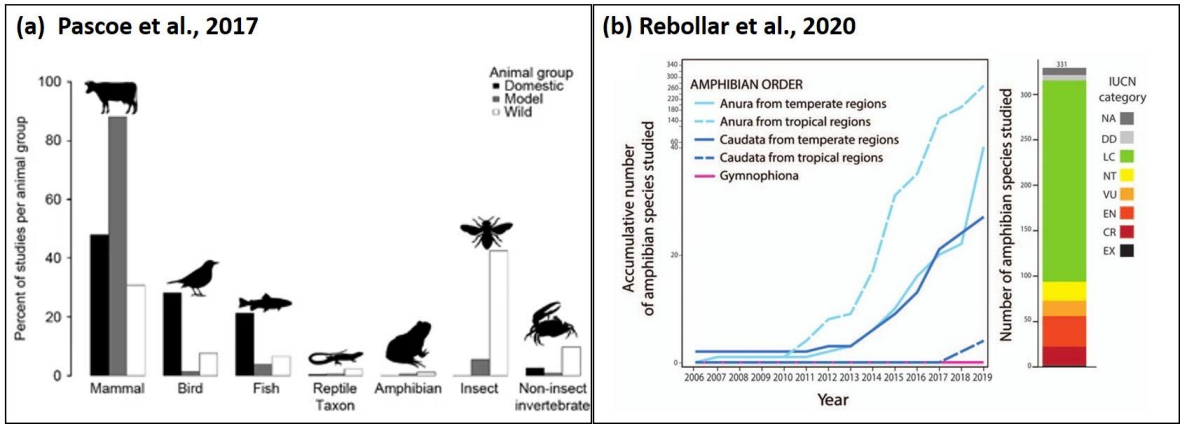

Figure 1. 4 . Overview of available literature on gut microbiome from different groups of organisms. Figure a retrieved from Pascoe et al (2017) and figure b retrieved from Rebollar et al (2020).

1.3.4. The amphibian microbiome

The number of studies addressing the amphibian microbiome greatly increased in the last years but are still significantly lower than works focusing on humans and other model organisms (Fig. 4a). Moreover, within amphibians, the studies have been highly biased towards species from tropical regions and mostly anurans (Fig. 4b).

Recent studies demonstrate that amphibian symbiotic communities can significantly differ depending on the host species, ontogeny, health status, host ecology, behavior, habitat type and environmental conditions (Jiménez & Sommer, 2017)(Fig. 5). Skin and gut are the two most commonly studied microbial assemblages in amphibians, due to their fundamental implications on host fitness and adaptation (Jiménez & Sommer, 2017).

- **Skin microbiome**

Amphibian's skin constitutes the first defense of the individual against environmental stressors and external threats such as dehydration or physical damage (Rooij et al., 2015). The skin presents an external layer of mucous that includes glycosylated mucins and mucopolysaccharides that gives the animal its characteristic moisture appearance and supports different types of constituents of the innate and acquired defense system, which include antimicrobial peptides (AMPs), alkaloids, bacteria that synthetized antifungal metabolites, lysozymes or antibodies (Varga et al., 2019). The skin is a very rich and dynamic environment and harbors a highly diverse community of microbes (e.g. bacteria,

archaea, fungi) that can be either mutualistic or pathogenic (Chen et al., 2018). The mutualistic microbes, especially bacteria, that are found associated to the skin of amphibians are important constituents of their metabolic and immunologic processes (Grice & Segre, 2011), with for example some bacteria reducing the growth of some specific microbial pathogens, such as *Bd* (Harris et al., 2006; Kueneman et al., 2016).

Following the patterns observed in other vertebrate groups, the skin microbiota of amphibians can vary with host species, individual characteristics, as well as with habitat features (Bletz et al., 2017; Kueneman et al., 2019; Mckenzie, Bowers et al., 2011a). When comparing the bacterial communities of several amphibian species of Madagascar, Bletz et al (2017a) found that host ecology was a major driver for microbial composition and had greater influence than host phylogeny (Bletz et al., 2017; Bletz et al., 2017b; Mckenzie et al., 2011a). At the same time, amphibian skin components (e.g., secretions, antimicrobial peptides (AMPs), alkaloids) have been found to affect skin microbiomes either by controlling colonization by external bacteria or inhibiting growth (Mina et al., 2015; Rollins-Smith et al., 2005). Some species have skin-associated bacteria rarely found in the surrounding habitat, suggesting that each host has its own specific microbial signature (Walke et al., 2014b), while some bacterial taxa seems to be coherently present across specific host species or population (core microbiome) (Loudon et al., 2014; Prado-Irwin et al., 2017). On the other hand, skin microbiota can be affected by the environmental pool of bacteria where the skin may actively filter and maintain specific bacterial taxa (Loudon et al., 2014) and in some cases selecting for rare environmental microbes (Walke et al., 2014b). It was observed that sympatric amphibian species can have similar skin taxa composition highlighting that the host effect is sometimes limited (Muletz Wolz et al., 2018), while in other instances has stronger effects than the habitat (Bletz et al., 2017b).

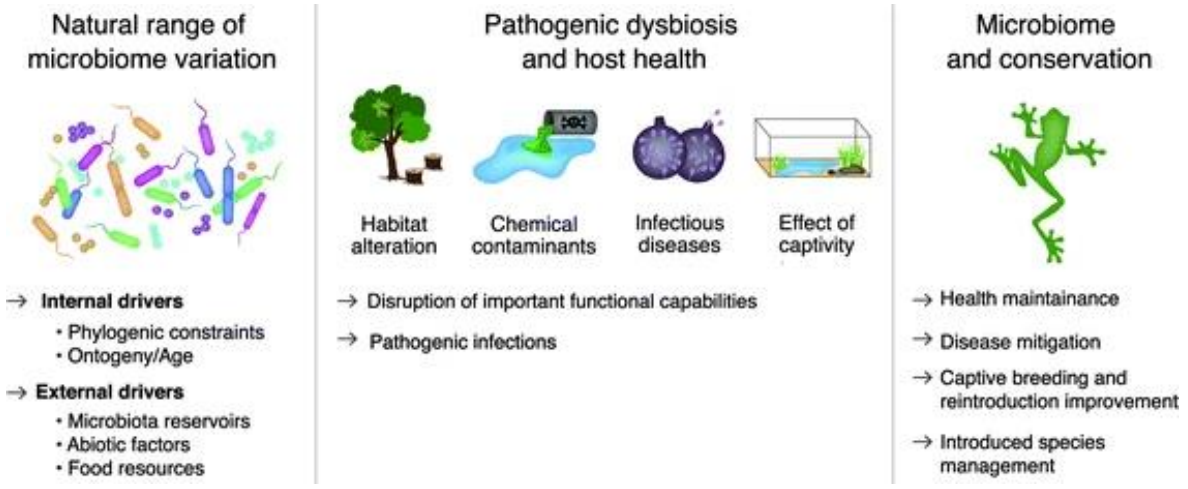

Figure 1. 5. Diversity of factors affecting amphibian microbiome. Figure retrieved from Jimenez and Sommer (2017).

- **Gut microbiome**

The gut microbiota of amphibians is less studied than the skin microbiota but similarly to other vertebrates, is generally characterized by great taxonomic and functional diversity (Jiménez & Sommer, 2017). Among the most abundant phyla found associated with the gut are the Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria, with fermentative bacteria known to have a key role in the host digestion (Colombo et al., 2015; Colston & Jackson, 2016). Evidence that gut bacteria differs between species and life stages has been previously observed, with tadpoles generally exhibiting higher bacterial diversity than the adults of the same species (Kohl et al., 2013; Vences et al., 2016; Warne et al., 2017), with diet type greatly influencing the gut symbiotic community (Vences et al., 2016; Youngblut et al., 2018). Hosts that present a carnivorous diet demonstrated a significantly different gut microbiota (in terms of taxa composition and phylogenetic richness) from the host with a herbivorous diet and this pattern has been consistently observed for other vertebrates (Ley et al., 2008; Youngblut et al., 2018). An experimental study where individuals were subjected to fasting exhibited drastic increase in microbial diversity in different hosts, with different patterns depending on the gut section (Kohl et al., 2014). The digestive system of anurans is known to suffer drastic restructuring during metamorphosis that is matched with different dietary preferences of the hosts (Hourdry et al., 1996). After metamorphosis, the adult' gut exhibit significant changes in community composition and in relative abundances of dominant phyla including an increase in Firmicutes abundance and a decrease in Proteobacteria and other phyla (Kohl et al., 2013). The dominance of Proteobacteria was observed in fishes indicating that tadpoles seem to exhibit a gut

community more similar to these group than to its adult terrestrial stages (Kohl et al., 2013; Sullam et al., 2012). On the other side, the gut community of adults resembled more the ones of amniotic organisms indicating a common patterns among different host groups (Ley, Hamady, et al., 2008). The physicochemical properties of the digestive system includes a simplified coiled tube in tadpoles, while adults have a fully functional gastric stomach and pH alterations that are both expected to affect their microbiomes (Kohl et al., 2013). Like the skin assemblages, also the gut microbiota has been found to be associated with several abiotic and biotic variables. These include the temperature that can affect bacterial taxa abundance (Kohl & Yahn, 2016; Sepulveda & Moeller, 2020), habitat alterations such as agricultural activities that can alter feeding habits by changing the availability of preys (Chang et al., 2016; Huang et al., 2018), or the position in the trophic chain (Liu et al., 2016).

1.4. Objectives and thesis chapters

Research focusing on host-associated bacteria increased exponentially over the last two decades, expanding our knowledge on the diversity, role and importance of these associations within the animal kingdom and more recently greatly expanding this research to include non-model organisms, while it is noted that microbial research is hampered by the complexity of factors affecting microbes communities which widener the research questions to be investigated (Antwis et al., 2017). As such, it is necessary to understand individual drivers of community composition as well as their interactions. Meta-analysis allows comparing the influence of several abiotic and biotic variables shaping host-associated communities, including the role of shared ancestry in shaping bacterial community compositions (Ross et al., 2019). Often, sampling natural populations is not adequate due to the multiple environmental variables affecting host and host-associated communities. In comparison, laboratory experiments are still scarce, and existing works focus mainly on model organisms. Similarly, the majority of the studies focus on adult individuals, and little is known on the host-associated microbiome changes during the decisive earlier life stages of amphibian's development. Finally, the characterization of the microbiome of an invasive amphibian species can provide new evidence on potential impacts on native co-occurring species and how the microbiome is affected by new surrounding environmental conditions.

In this thesis, I address questions related to the effect of ecological settings and developmental stages in shaping host-associated microbial communities in different amphibian species, and attempt to contribute towards an integrative understanding of the

amphibian microbiome development. In chapter I, I explored the skin microbiome responses to human disturbance by comparing the effect of habitat alterations (from pristine forest to human altered areas) in co-occurring amphibian hosts in Madagascar. In chapter II, I focused on the characterization of the microbial diversity of an invasive species across its incursion area in Madagascar, comparing it to the microbial diversity observed in a co-occurring native species. In chapter III, I compared the microbiome changes during metamorphosis on co-occurring anurans and urodele species that are sharing the same spatial and temporal window. This experimental design allowed controlling for confounding environmental effects and focus on species-specific factors such as identity and developmental stage. Finally, in chapter IV, I performed a simulation of an alteration in the aquatic environment, exposing the tadpoles of two species to both native and translocated waters, and assessed the effects on their microbiome. This allowed to control the surrounding environmental parameters (e.g., temperature, light, diet, etc.). With this experiment, we aimed to understand the capacity of colonization of new bacteria from new water sources in each species. With exception of chapter I, where we sampled only skin swabs of native species, in the remaining three chapters we compared both skin and gut bacterial communities, a comparison still rare in microbial studies.

This thesis is organized by chapters written in format of research articles, which will be subjected to future publication with one (Chapter III) already submitted for peer review.

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CHAPTER II

Exploring the composition of amphibians' skin bacteria from sub-pristine and human-impacted habitats in eastern Madagascar

Paper in preparation

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ABSTRACT

In Madagascar, large portions of forested habitat have been transformed into rice fields and pastures. Only a few amphibian species occur in both natural and highly anthropized habitats. We characterize the skin bacterial community of two amphibian species occurring in distinct microhabitats with different level of human impact and in waterbodies with different pH levels. Overall, the habitat type seems not to affect the skin bacteria, however, individuals collected from more basic waters within the more impacted habitat (e.g., Rice fields) type are associated with poorer skin bacterial communities. Impoverished bacteria richness is accompanied by a higher percentage of bacteria with anti-*Batrachochytrium* dendrobatidis (a parasitic fungus associated with global amphibian declines) properties, indicating that water pH may influence host-defense capacity by mediation of the skin microbial community.

Keywords 16S rRNA, microbiota, water pH, anthropogenic impact, rice fields, Andasibe

INTRODUCTION

Microbes, such as bacteria, play a crucial role in the environment and ecosystem functions and are found associated with all types of habitats and environmental layers (e.g., water, soil, vegetation, among others), but also in symbiotic relationships with other organisms. In amphibians, skin-associated symbiotic bacteria have been widely studied over the last decade due to their diversity and implications in host-disease resistance and other physiological functions (Bletz et al., 2017b; Kueneman et al., 2014; Woodhams et al., 2007). Bacteria can provide support to the amphibian host to fight pathogens invasion or to cope with less suitable habitat alterations (Costa et al., 2016; Longo & Zamudio, 2016). The disruption of the skin bacterial community (either by losing some taxa, functional diversity or overwhelmed by occurrence of pathogenic taxa) may increase amphibians' susceptibility to pathogens and diseases (Piovia-Scott et al., 2017). Among the most common amphibian pathogens, the chytrid fungus *Batrachochytrium dendrobatidis* (herein called *Bd*) has been detected worldwide and it is responsible of population declines in several species across the globe (Scheele et al., 2019). *Bd* incidence has also been related to host characteristics as well as climate conditions or water properties such as pH level (Kärvemo et al., 2018). In Madagascar, *Bd* has been detected in multiple localities and in individuals from all native anuran families (Bletz et al., 2015). Despite this, recent studies using *in vitro* assays have

shown that some amphibians, including some Malagasy species, harbor skin bacteria with anti-*Bd* properties (Piovia-Scott et al., 2017).

The amphibian skin bacteria can differ by host species, population and environmental gradients, but it is sometimes difficult to disentangle single effects among the several co-occurring biotic and abiotic factors (Hughey et al., 2017; Kueneman et al., 2014). Skin-associated microbial communities can be greatly influenced by the environment microbial pool (e.g., either in the water or soil), while host intrinsic characteristics, such as skin properties or ecological requirements, can influence the exposure to new bacteria by either favoring or blocking their colonization (Mckenzie et al., 2011a; Walke et al., 2014b).

Madagascar harbors one of the richest amphibian community worldwide, characterized by a high number of species with a wide range of life history traits (Vences et al., 2009; Vieites et al., 2009). Over the last decades, Madagascar experienced a rapid increase in deforestation, and several pristine or semi-pristine areas have been transformed into rice fields (Harper et al., 2007;Vieilledent et al., 2018; Zaehringer et al., 2015). Environmental alterations such as conversion of forest landscapes to rice fields can cause disturbances in habitat quality and potentially lead to an alteration in the environmental pool of bacteria. Environmental microbes have been found to be commonly affected by a wide range of habitat parameters (e.g., temperature, pH, nutrients, among others) (Lauber et al., 2009; Rousk et al., 2010). Amphibia76oiklns occurring in habitats with different water or soil pH may also exhibit differences in the skin-bacterial communities in parallel with alterations in the environmental pool of bacteria (Chang et al., 2016; De Assis et al., 2017; Hughey et al., 2017).

Due to the historical and ongoing increase of destruction of natural forests, it is fundamental to understand how skin-associated bacterial communities of amphibians may be affected by habitat alteration by comparing semi-pristine areas with more anthropized habitats. In this work we characterize the skin microbiome of two endemic frog species occurring in semi-natural and anthropized habitats, and explore the effect of water pH on their bacterial community.

MATERIALS AND METHODS

Sampling

We sampled two amphibian species: *Ptychadena mascareniensis* is a widespread and abundant frog species occurring from sea level areas up to ca. 2000m of altitude. It can be found in stagnant and sun exposed water bodies in agricultural (including rice fields and urban areas) and more pristine areas (including the edges of rainforests, in clearing in rainforest and in dry lands). *Mantidactylus betsileanus* is generally found along slow-moving streams in rainforest habitats, but can sometimes be observed in more disturbed habitats, such as rice fields or in close proximity to forest edges (AmphibiaWeb 2019, n.d.).

Fieldwork was conducted within and around Analamazaotra forest, a forest fragment bordering Andasibe-Mantadia National Park, and located South of the village of Andasibe, in central Madagascar, at about 930–980 m altitude (18°56.288' S, 048°24.851' E; Fig. 2.1). We selected 3 habitat types and sampling was performed at several sites within each microhabitat: “Rice field” habitat included 5 sites; “Border” habitat (sites located between the rice fields areas and Analamazaotra forest) included 4 sites; “Sub-pristine” habitat included 3 sites of dense forest and 3 clearings within the forest and sample the two species across the habitats (Fig. 2.1, Table 2.1).

Each specimen was collected with new nitrile gloves and kept in individual sterile plastic bag. Specimens were sexed and only adult males were used for further analyses. Each specimen was rinsed with 50 mL of sterile water to wash off transient microbes and swabbed 20x over the ventral surface (10x on the stomach, 5x on each thigh and foot) using a tubed sterile dry swab with a fine tip (MW100). Samples were maintained in cool condition (ca. 4ºC) until their export and further processing. A swab from the water was also collected for each site. The pH value of each sampling site was measured in triplicate using a portable pH meter, Extech EC510 (Table 1 shows average value for each site). Research permits were provided by Malagasy authorities, research permit: 223/15/MEEMF/SG/DGF/DAPT/SCBT.

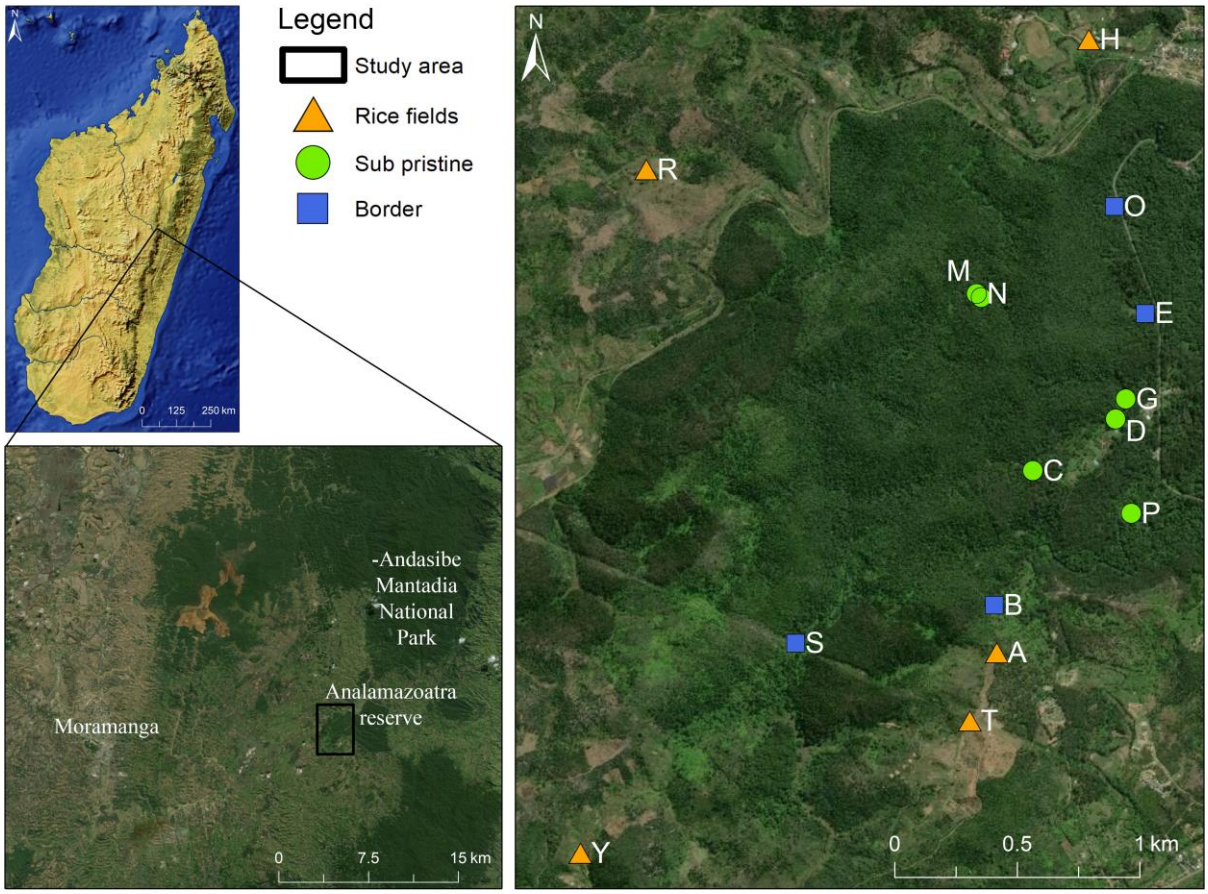

Figure 2. 1. Sampling sites classified as Rice field (orange triangles), Sub-pristine (green circles) and Border (blue squares) close to Analamazoatra Reserve.

DNA extraction, Amplification and Sequencing

Total DNA was extracted from the swabs using a QIAGEN DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Before extraction, swabs were pre-treated with a combination of lysozyme and lysis buffer to break up the Gram-positive bacteria's cell walls. The V4 region of the bacterial 16S rRNA gene was amplified using a dual-index approach with barcode primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (10µM) (Caporaso et al., 2012). The amplification was done in duplicates in a volume of 12.5 µl using 0.2 µl of Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, Ma, USA), 0.25 µl of each primer (10 µM), 0.25 µl of dNTPs, 2.5 µl of buffer, 8.1 µl of H2O and 1 µl of template DNA. The amplification protocol consisted of an initial denaturation step at 98ºC for 1 min, followed by 30 cycles of denaturation at 98ºC for 10 s, annealing at 55ºC

for 30 s and elongation at 72ºC for 30 s, with a final extension at 72ºC for 5 min. The PCR products were pooled in a total volume of 25 µl, visualized on 1% agarose gel and purified with QIAQuick Gel Extraction Kit (Qiagen, Hilden, Germany). Successfully amplified samples were sequenced with paired-end 2 x 250 v2 chemistry on an Illumina MiSeq sequencer (Kozich et al., 2013).

Sequence Processing

All data was processed using QIIME2 (Quantitative Insights into Microbial Ecology, (Bolyen et al., 2019; Caporaso et al., 2010)). Only the forward reads were used after filtering using the following criteria: no Ns within the sequence allowed, no barcode errors or presence of three or more consecutive low-quality base pairs. Sequences were trimmed to 150 bp, excluded if had less than 10 reads and clustered into Sub-Operational Taxonomic Units (sOTUs) following the Deblur workflow (Amir et al., 2017). The resulting sOTUs were assigned to a taxonomy using the Greengenes 13.8 reference database (May 2013 release; http://greengenes.lbl.gov/). Non-bacterial taxa (e.g.: Mitochondria and Chloroplast) were removed from the dataset. PyNAST (Caporaso et al., 2010) was used to align the sOTUs representative sequences, and a phylogenetic tree was built with FastTree (Price et al., 2010). The final sOTUs comprising less than 0.001% of the total reads were excluded from the further analysis and all samples were rarefied to 1000 sequences per sample.

Table 2. 1. Sampling habitats and sites with respective water pH values, latitude and longitude coordinates and total number of collected samples of *Ptychadena mascareniensis*, *Mantidactylus betsileanus* and water swabs used for the analysis.

Habitat type	Habitat variation	Site	Water pH	Latitude	Longitude	*Ptychadena mascareniensis*	*Mantidactylus betsileanus*	Water
Rice fields		A	7.57	18.94535	48.40838	4	4	1
		H	7.83	18.92282	48.41176	2	0	1
		R	6.39	-18.9276	48.39551	4	0	1
		T	7.42	18.94786	48.40739	3	0	1
		Y	8.36	18.95272	48.39309	5	0	0
Border		B	6.96	-18.9436	48.4083	2	3	1
		E	7.55	18.93288	48.41384	0	6	1
		O	6.68	18.92895	48.41271	0	3	1
		S	6.91	18.94508	48.40136	0	2	0
Sub pristine	**Clearing within Forest**	C	6.95	18.93867	48.4097	2	2	0
		D	7.21	18.93677	48.41274	0	5	0
		P	5.92	18.94023	48.41332	0	2	0
	Forest	G	7.23	18.93603	48.41312	0	7	1
		M	5.65	-18.9323	48.4078	0	8	1
		N	7.44	18.93216	48.40764	0	2	1

Data analysis

All the statistical analyses were performed in R 3.6.1 (R Core Team, 2019). Data was divided into two datasets. Dataset A includes all the samples collected from all the 15 sampling sites and grouped into the three habitat types (Sub-pristine, Border and Rice fields). This dataset was used to assess the effects of host species and habitat type on the skin bacterial communities. Dataset B includes individuals of *P. mascareniensis* from the five Rice field sites and the individuals of *M. betsileanus* from the three sub pristine sites and was used to assess the effects of water pH in each species that were analyzed separately. For Dataset A, species richness (as number of sOTUs) and Faith's phylogenetic diversity (herein called PD) of the skin bacterial communities were compared by fitting data to linear mixed-effects models using maximum likelihood (lme4::lmer(); (Bates et al., 2015)), and we test for an effect of species and habitat type with site (nested within habitat type) as random factor to control for site-specific variation. For Dataset B, the same diversity metrics were calculated for each species individually by using each site (representative of water pH value) and a non-parametric Kruskal-Wallis multiple comparison test with post-hoc Dunn test for pair-wise comparisons and correction of p-values using Bonferroni adjustment method (Dinno, 2015)

The community composition (Beta diversity) was assessed using Permutational multivariate analysis of variance (PERMANOVA, function adonis, 1000 permutations) on unweighted Unifrac distance matrix (Lozupone & Knight, 2005) to determine if Beta diversity was better explained by host species, habitat type (Dataset A) or water pH within habitat (Dataset B). When permanova test indicated a significant variation explained by one of the factors, a post-hoc pairwise comparison test was performed using Tukey's honest significant statistics and controlling for the false discovery rate of multiple comparisons (Y & Y, 1995). For the Dataset B, the total of unique and shared bacterial OTUs among individuals of the same species from sites with different water pH was also calculated. The final OTUs in Dataset B were also mapped against the database of antifungal amphibian skin bacterial isolates that includes isolates that are likely able to inhibit the growth of the amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*) (Woodhams et al., 2015). A match of ≥97% sequence similarity was selected and the proportion of *Bd*-inhibitory bacteria was computed for each species at each site. All data was plotted using the following ggplot2 package (Villanueva & Chen, 2019).

RESULTS

The number of collected specimens from each species at each site (organized in the three habitat types) is available in Table 1. *Ptychadena mascareniensis* was more abundant in rice field habitat, and only two individuals were collected in border and sub-pristine sites. On contrary, *Mantidactylus betsileanus* was more abundant in the sub-pristine and border sites and only four individuals were collected in the rice fields.

Diversity of skin bacterial communities across species and habitats (Dataset A)

OTU richness did not differ between species or habitats or due to their interaction (Species: X^2=2.81; Habitat: X^2=0.58; Species*Habitat: X^2=2.91, all p>0.2; Figs. 2.2A, S2A, Table S1). Bacterial communities of species and habitats did not differ also in terms of phylogenetic diversity (Species: X^2=1.44; Habitat: X^2=0.80; Species*Habitat: X^2=1.42, all p>0.4; Figs. 2.2B, S2B, Table S1). However, some trends could be observed (Fig. 2A-B), with both species from border sites having higher average values for alpha diversity; bacterial communities occurring in the water had higher average values (alpha diversity) in rice field sites; and *P. mascareniensis* seemed to harbor richer skin communities than *M. betsileanus* (Fig. 2.2A-B).

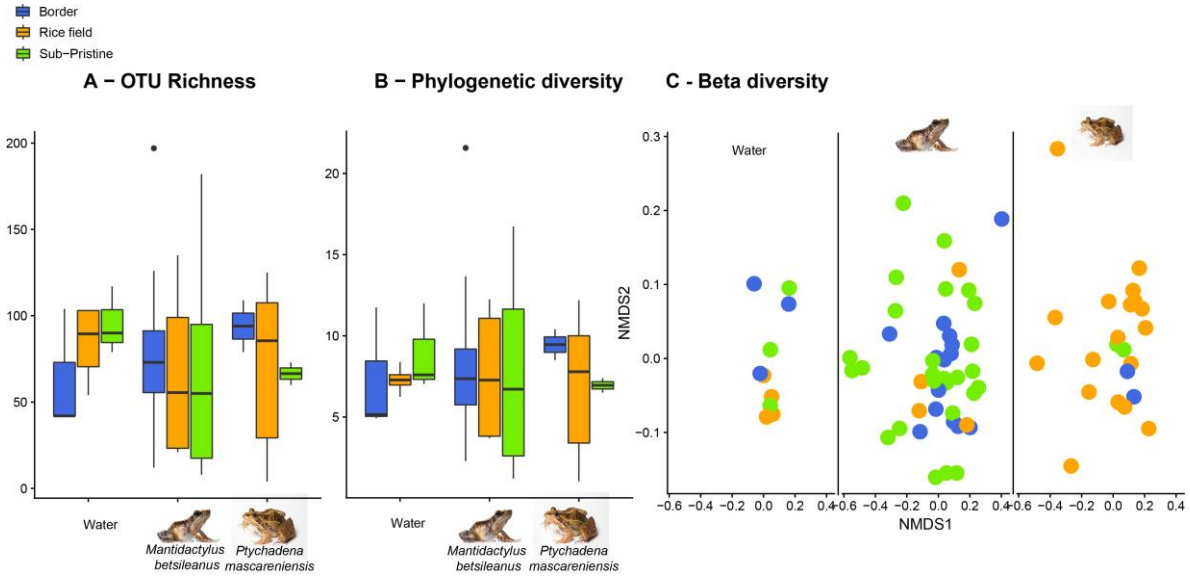

Figure 2. 2. Alpha and Beta diversity. Alpha diversity is represented as A) OTU richness and B) Phylogenetic diversity and Beta Diversity (C) of skin bacterial from individuals of *Mantidactylus betsileanus*, *Ptychadena mascareniensis*, and water samples from each habitat.

Community structure (Beta diversity) did not differ among species or habitats (PERMANOVA: Species: Pseudo-F=1.09, R2=0.03, p=0.29; Habitat: Pseudo-F=1.04, R2=0.03, p=0.43; Species*Habitat: Pseudo-F=0.93, R2=0.05, p=0.64; Figs. 2.2C, S2C, Table S3). In terms of composition, the skin communities from all groups were primarily composed by four major phyla (e.g., Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria), with differences in the relative abundances across species and habitats (Fig. 2.3). Proteobacteria was more abundant in *P. mascareniensis* individuals from rice fields and border habitats but lower in individuals from sub-pristine habitats. Individuals of *M. betsileanus* collected at rice fields sites exhibited a skin community dominated by Bacteroidetes contrasting with the remaining groups that were dominated by Proteobacteria.

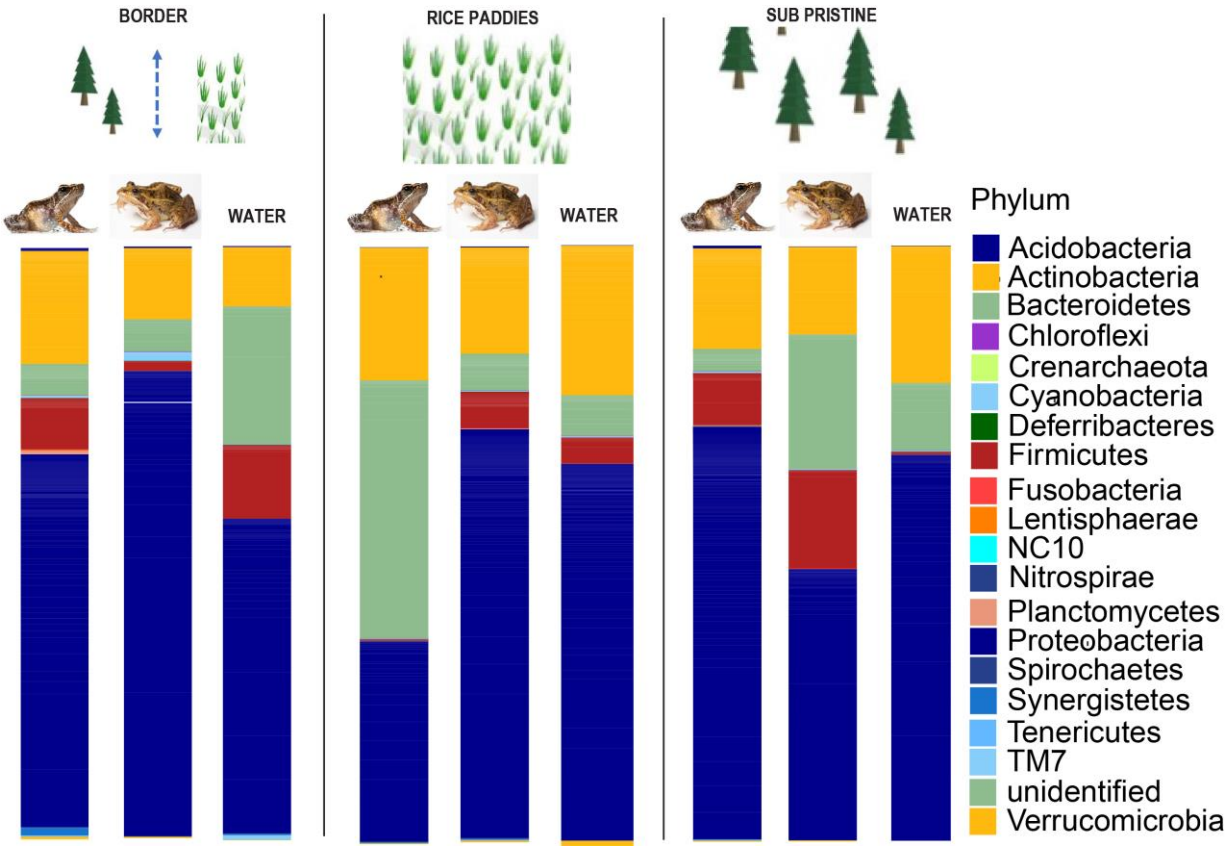

Figure 2. 3. Relative abundance of the 20 most common taxa at Phylum level occurring in the skin of *Mantidactylus betsileanus* (left), *Ptychadena mascareniensis* (center) and water samples (right) from Border, Sub Pristine and Rice field habitats.

In terms of composition of the most abundant bacteria classes, the majority of the groups were dominated by Gammaproteobacteria, followed by Alphaproteobacteria, Actinobacteria, Sphingobacteriia, Betaprotebacteria and Bacilli with differences at the abundance levels among groups (Fig. 2.4).

The most common (abundant) family was Pseudomonadaceae, while Xanthomonadaceae was the most homogenous across groups. Several other families exhibited differences in the abundance levels (Fig. 2.5). The two host species also exhibited higher relative abundances of Cellulomonadaceae when collected at the sub-pristine areas, following the same pattern observed for the water (Fig. 2.5).

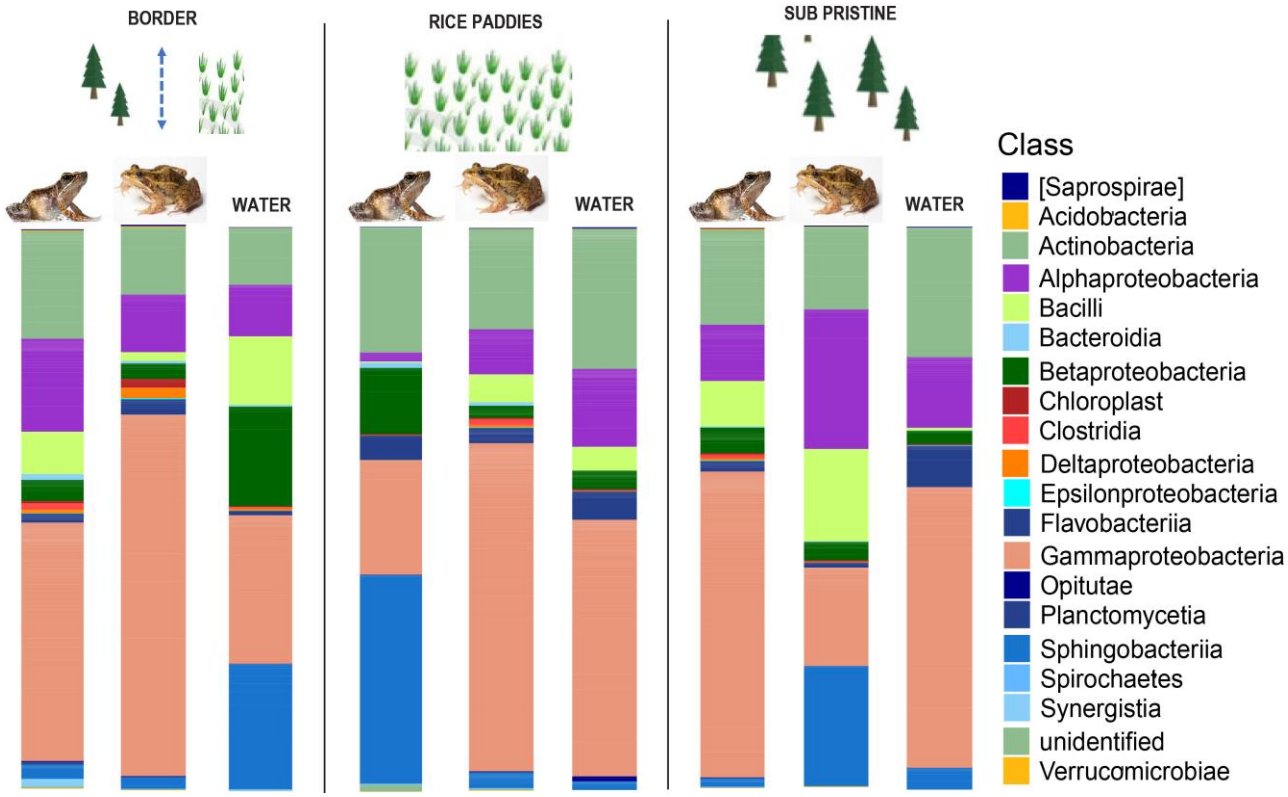

Figure 2. 4. Relative abundance of the 20 most common taxa at Class level occurring in the skin of *Mantidactylus betsileanus* (left), *Ptychadena mascareniensis* (center) and water samples (right) from Border, Sub Pristine and Rice field habitats.

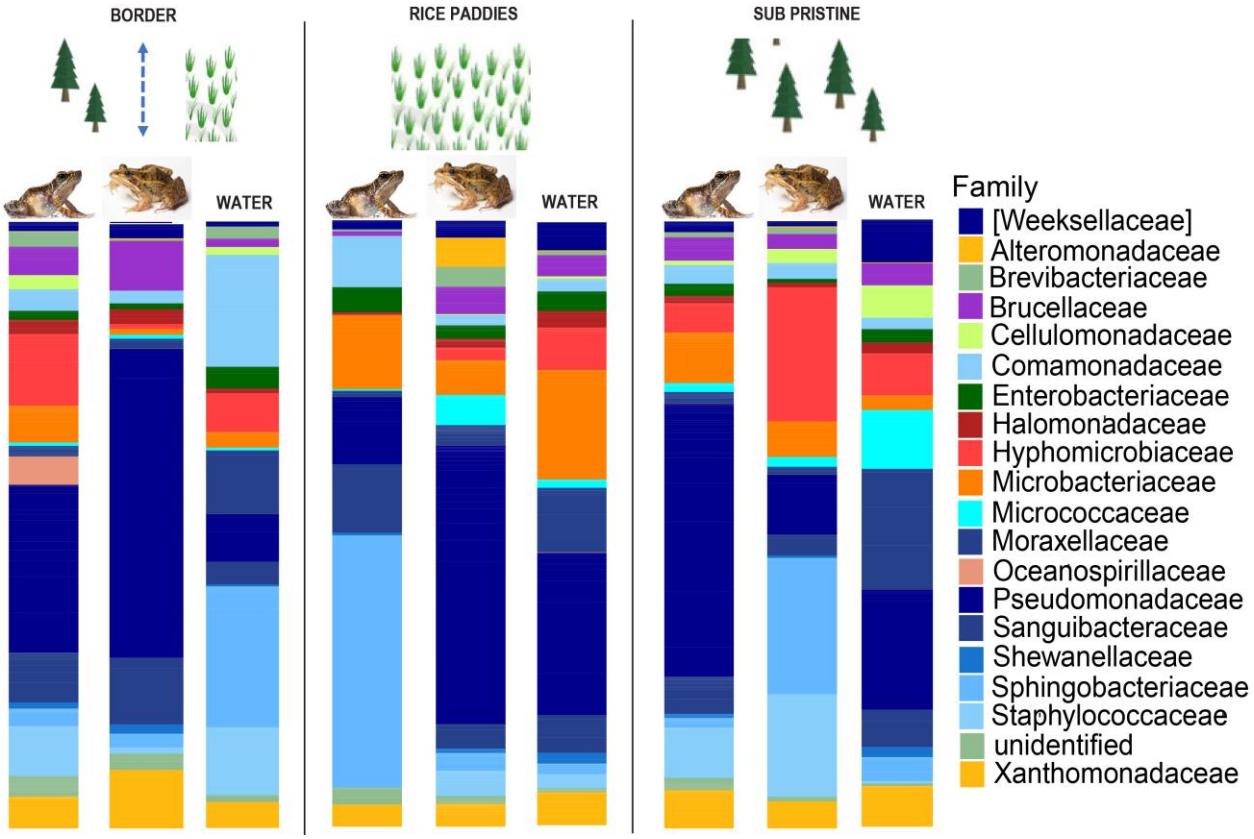

Figure 2. 5. Relative abundance of the 20 most common taxa at Family level occurring in the skin of *Mantidactylus betsileanus* (left), *Ptychadena mascareniensis* (center) and water samples (right) from Border, Sub Pristine and Rice field habitats

Effects of pH on skin-associated bacterial community of amphibians (Dataset B)

Two sub-datasets were used: 1) individuals of *P. mascareniensis* collected in five sites from rice fields habitat with increasing water pH; 2) individuals of *M. betsileanus* collected in three sites from sub-pristine habitat with increasing water pH.

- ***Ptychadena mascareniensis* occurring in Rice field habitat**

Significant differences were found between individuals from sites with different water pH levels (Fig. 2.6A-B): individuals from the site with the highest water pH (8.3) harbored significantly poorer skin communities both in terms of OTU richness (K-W: p=0.02) and phylogenetic diversity (K-W: p=0.005) in comparison with individuals from the site with the lowest water pH, that harbored the richer bacterial communities.

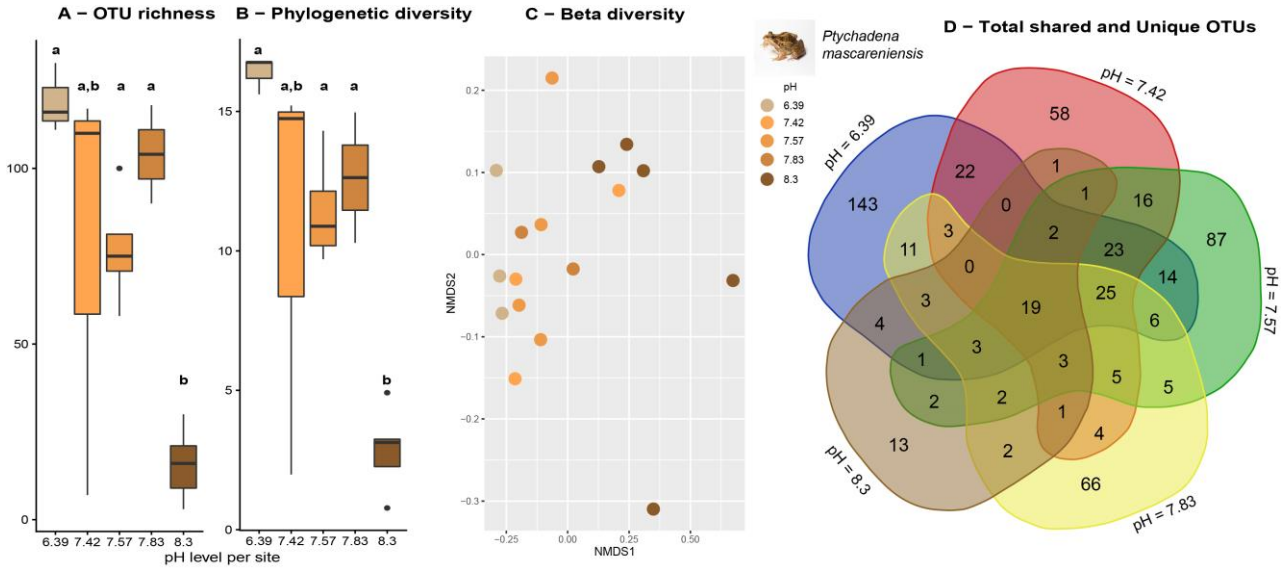

Figure 2. 6 Diversity of skin-associated bacteria in *Ptychadena mascareniensis* sampled across five rice field sites with increasing water pH level: A) OTU richness; B) Phylogenetic diversity; C) Beta diversity; D) Total of shared and unique OTUs. In A and B, groups

The water pH explained 35% of variation of the skin bacterial composition of *P. mascareniensis* individuals (PERMANOVA: F= 1.60, R^2=0.35, p<0.001, Fig. 2.6C) although this effect cannot be dissociated from the site identity, since we only had one site with such low water pH. In terms of relative abundance of the most prevalent bacterial groups, the class Gammaproteobacteria and the family Pseudomonadaceae were the most abundant taxa in the skin-associated bacteria from the waters with the highest pH from site Y (Fig. 2.7). The high abundance of the Pseudomonadaceae family found in individuals collected from site Y was mainly derived from two OTUs with the most abundant corresponded to the genus *Pseudomonas* and observed in several individuals of *P. mascareniensis* and *M. betsileanus* across the dataset A and B, however, it occurred in high abundances only in *P. mascareniensis* from site Y (Fig. S3).

P. mascareniensis individuals collected at the five sites shared a total of 19 skin OTUs and exhibited a decreasing number of unique OTUs from the most acidic site (143 unique OTUs = 51% of total OTUs) to the most basic site (13 OTUs = 23% of total OTUs) (Fig.2.6D). On the contrary, we observed an increase in the proportion of *Bd*-inhibitory OTUs in individuals from the most acidic (20%) to the most basic water (41%) (Table 2.2).

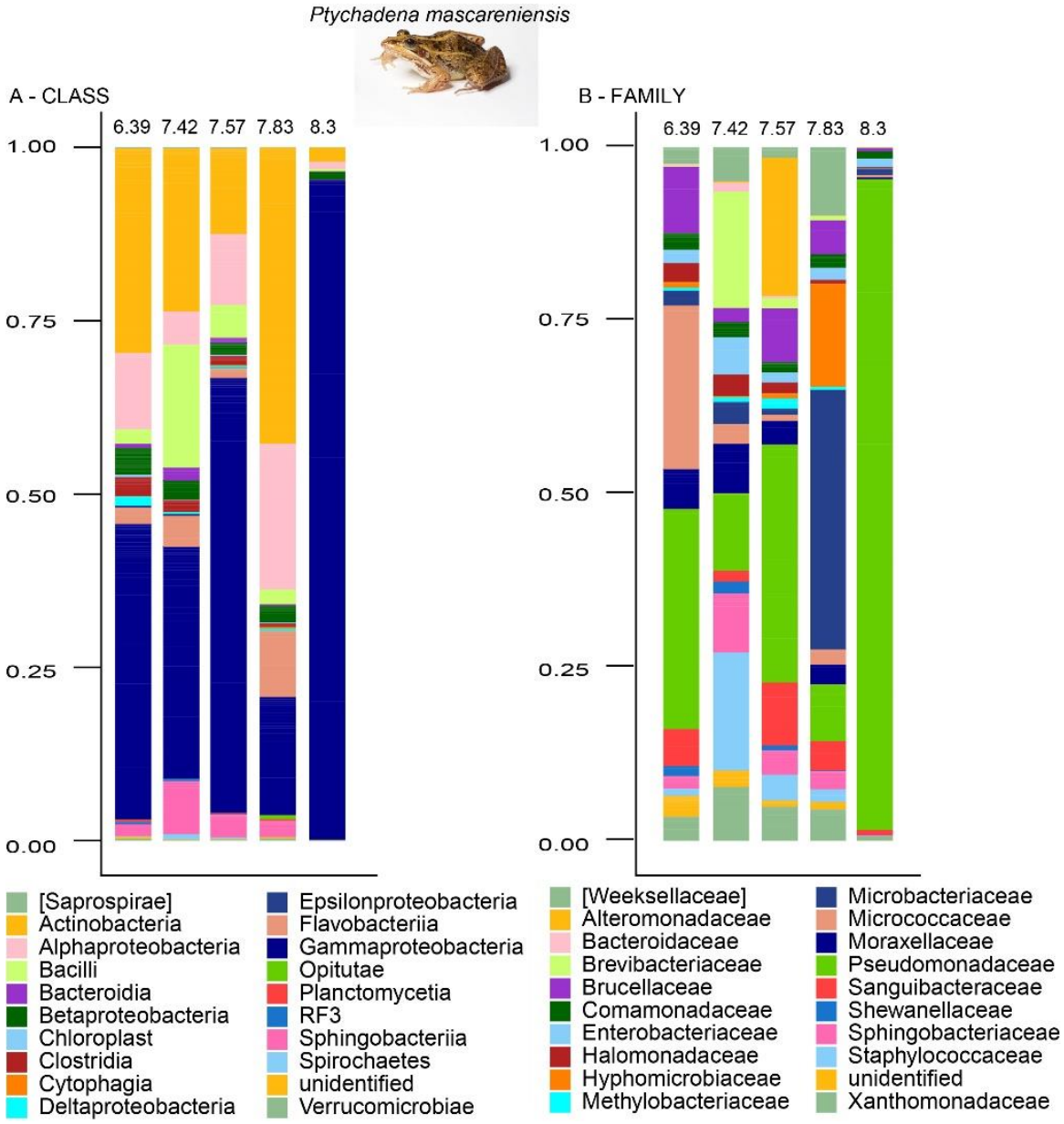

Figure 2. 7. Relative abundance of taxa of skin-associated bacteria in sampled individuals of *Ptychadena mascareniensis* from five rice field sites with increasing water pH levels: A) Class; B) Family

- ***Mantidactylus betsileanus* occurring in sub-pristine habitat**

Contrary to what it was observed for *P. mascareniensis*, no differences in alpha diversity metrics or bacterial structure ($p>0.05$) were observed between individuals from the three sub-pristine sites with different water pH (Fig. 2.8A-C). However, similarly to *P. mascareniensis*, also in individuals of *M. betsileanus*, Gammaproteobacteria was found to be the most abundant skin-associated bacterial class, followed by Actinobacteria and

Alphaproteobacteria, the latter exhibiting an increase in relative abundance with increasing pH levels (Fig. 2.9).

Different from what it has been observed in *P. mascareniensis,* where Gammaproteobacteria and Pseudomonadaceae were more abundant in basic waters, the class Gammaproteobacteria and the family Pseudomonadaceae were found to be slightly more abundant in individuals of *M. betsileanus* from the most acidic water (pH= 5.65), while more basic waters exhibited higher abundances of Hyphomicrobiaceae, Sanguibacteraceae and Xanthomonadaceae (Fig. 2.9).

The individuals from the three sites shared a total of 36 skin OTUs (Fig. 2.8D). Similar to what it has been observed for *P. mascareniensis* (Fig. 2.6D), it was observed a decrease in the number of unique skin' OTUs in *M. betsileanus* individuals sampled from sites with increasing pH levels (Figs. 2.6D vs 2.8D). Moreover, the proportion of *Bd*-inhibitory OTUs increased from the site with more acidic water (19.3%) to the site with the most basic water (25.3%) following the same pattern observed for *P. mascareniensis* (Table 2.2).

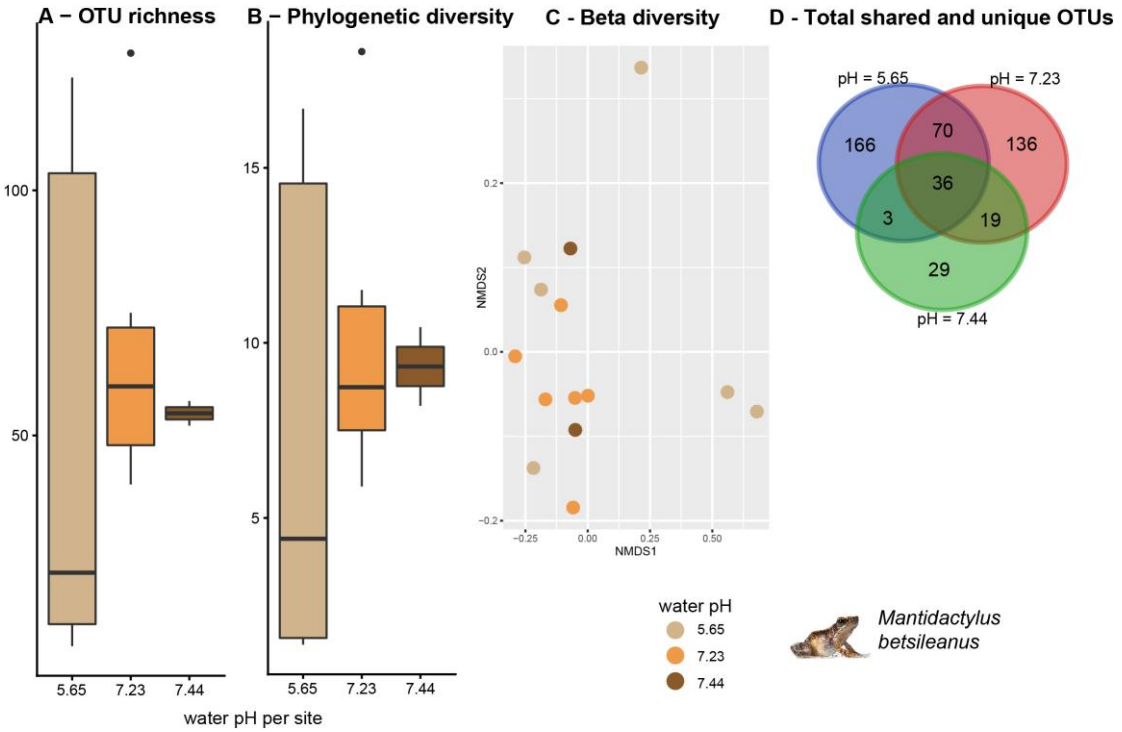

Figure 2. 8. Diversity of skin-associated bacteria of individuals of *Mantidactylus betsileanus* from three sub-pristine sites across a gradient of increasing water pH levels: A) OTU richness; B) Phylogenetic diversity; C) Beta diversity; D) Total of shared and unique OTUs.

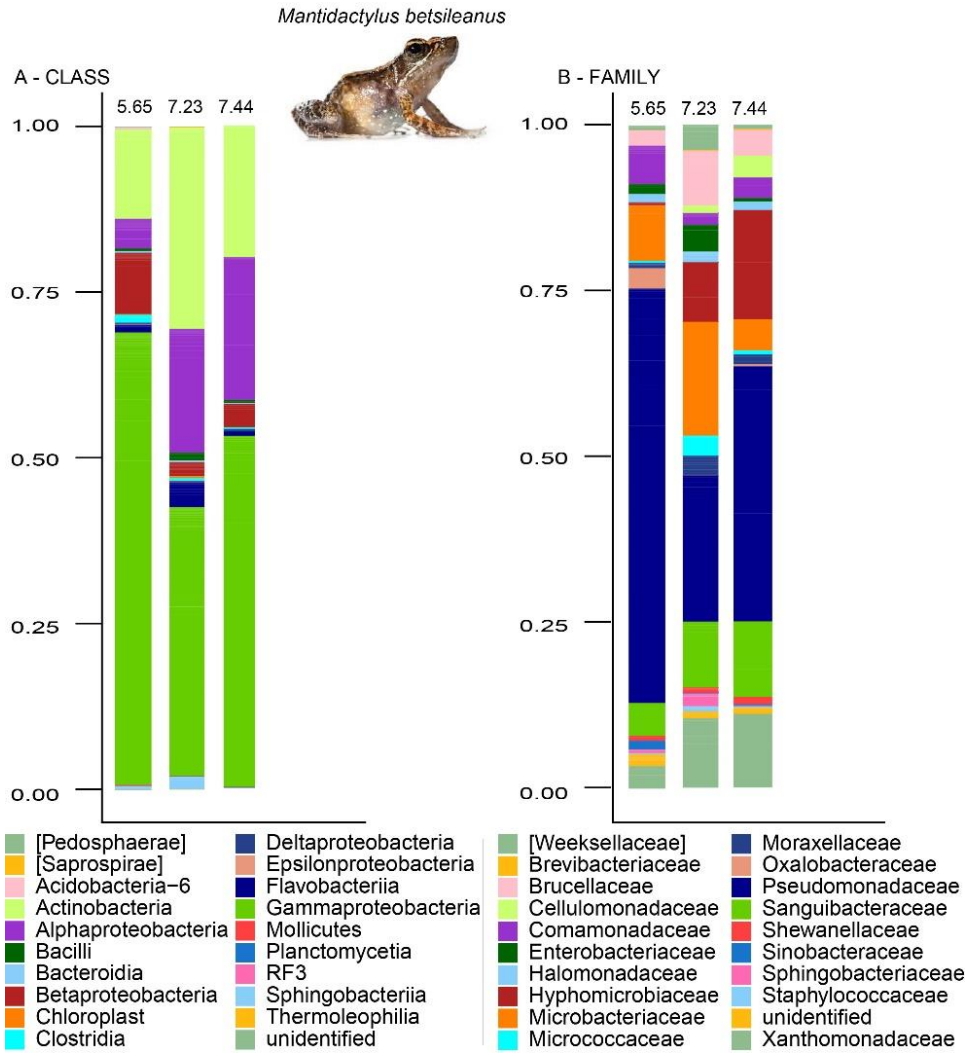

Figure 2. 9. Relative abundance of skin-associated bacteria in *Mantidactylus betsileanus* individuals from three sites with increasing water pH levels: A) Class; B) Family

Table 2. 2. Table showing water pH for each site, total number of samples of *Ptychadena mascareniensis* and *Mantidactylus betsileanus*, total of OTUs per group, total of Bd-inhibitory OTUs per group and percentage of Bd-inhibitory OTUs from sites with increasing pH

	Ptychadena mascareniensis					*Mantidactylus betsileanus*		
Site	R	T	A	H	Y	M	G	N
Water pH	6.39	7.42	7.57	7.83	8.3	5.65	7.23	7.44
Total of individuals	4	3	4	2	5	8	7	2
Total of OTUs	279	183	214	158	57	275	261	87
Total of inhibitory OTUs	56	43	50	42	24	53	57	22
% of inhibitory OTUs	20.1	23.5	23.4	26.6	42.1	19.3	21.8	25.3

DISCUSSION

Madagascar has been subject to an intense deforestation pressure to increase the area for rice cultivation and pasture (Harper et al., 2007; Vieilledent et al., 2018; Zaehringer et al., 2015). Although this is not widely studied yet, it is expected that this phenomenon will increase the vulnerability of Malagasy amphibian fauna to numerous environmental threats, including an increase in exposition to pathogens, and population decline due to the lack of suitable niches and habitat quality degradation (Ndriantsoa et al. 2017; Riemann et al., 2017; Vallan, 2000, 2002; Vallan et al., 2004). In the present work we explore the effects of habitat type (e.g., sub-pristine, border and rice field) on the skin-associated bacterial communities from two amphibian species from eastern Madagascar. At the same time, we also assessed if variation in water pH is affecting the skin-associated bacteria communities in the two species.

Apart from some differences in taxa abundance levels, it seems that habitat type is not significantly affecting the symbiotic communities from the skin of the selected host species. This is in accordance with a previous published work that reported the absence of differences when comparing the skin-associated bacterial communities in a generalist frog species from intact and disturbed forests (Hughey et al., 2017). The target species of this study are not generalists, and in fact our study reflects some ecological preference for each species, with the occurrence of *M. betsileanus* individuals being rare in rice fields and *P. mascareniensis* in sub-pristine habitats. The surveyed sites were located relatively close to each other, which could also contribute to this lack of differences (Fig. 1). The small geographic scale and the occurrence of each species in more than one habitat can help explaining the homogeneous skin bacterial community found. Unfortunately, the low sample size may have contributed to cover possible patterns since some trends could be observed in our results. For example, the two species exhibited higher average values for alpha diversity when occurring in the border sites (even with the difference in the sampling size), a habitat type that may exhibit higher variation in environmental conditions providing increased probability of exposure to different environmental bacterial pools and therefore, increasing the probability of colonization of the skin by new bacteria. *M. betsileanus* individuals collected in rice field exhibited a high abundance of Bacteroidetes, a cosmopolitan bacterial group that occur in almost all habitats, and includes some pathogenic taxa and other taxa that are known for their specialized capacity to degrade organic matter (Thomas et al., 2011). This higher abundance could be the result of the host receiving more

bacteria due to the water characteristics of the rice fields, which most likely contains higher levels of organic compounds (Li et al., 2018) and were contrasting with the lower abundance levels in individuals from sub-pristine areas. Indeed, when we check the most abundant taxa at the family level, the individuals' skin was dominated by Sphingobacteriaceae, a group previously associated with successful recovering from *B. dendrobatidis* in other frog species (Becker et al., 2015). Similarly, water samples from rice fields exhibited a tendency for a richer bacterial community comparing with the border and sub-pristine habitats. Rice field are agroecosystems often associated with high microbial biodiversity levels but also linked to alterations in water quality such as lower pH and increased levels of nutrients due to fertilization practices (Bambaradeniya et al., 2004; Li et al., 2018; Yuan et al., 2013).

Despite the lack of a "habitat" effect in shaping the skin associated bacterial communities in these two Malagasy amphibians, we observed significant differences in relation to water pH in one species (*P. mascareniensis*), with decreased OTUs richness and phylogenetic diversity in the most basic water, similar to what it has already been reported in previous works in amphibians associated to both aquatic or soil layers with variation in pH values (Varela et al., 2018). Despite pairwise comparisons showed that only one of the sites (with higher pH) was significantly different from the others, therefore indicating that the overall significance is mainly driven by that population, all the individuals carried consistently lower alpha diversity values comparing with individuals from the other sites. Although we cannot completely linked this difference to be derived only from water pH, since other site-specific environmental variables could be exerting a role, water pH is expected to be an important influence on amphibians skin microbiome as observed before (Krynak et al., 2015). The pH has been found to contribute to influence environmental bacterial communities as well as symbiotic communities in amphibians (Li et al., 2018; Varela et al., 2018). This highlights the importance of assessing water parameters and quality and the need to distinguish single effects of different environmental factors that may shape amphibians skin communities in different ways. Other studies found that amphibian larval stages exhibited marked alterations in the skin-associated bacteria when the water pH differed from 6 to 7 (Krynak et al., 2015) indicating that the water pH may be an important factor in shaping the skin communities across the amphibian development.

However, it is known that host identity also influences its response to environmental triggers and different species may be involved in different selection processes to what concern skin-associated bacteria (Bletz et al., 2017b). Interestingly, while in more pristine sites, *M.*

betsileanus may be exposed to poorer bacterial variation in the water environment, in more impacted habitats *P. mascareniensis* may be subject to more drastic changes in skin-associated bacterial. This might be a response to pesticide exposure (which are known to affect biodiversity at different levels including bacterial diversity (Muturi et al., 2017)), or the basification of water and soils in this habitat. *P. mascareniensis* individuals from more basic waters were found to have a skin-associated bacterial community dominated by Gammaproteobacteria, especially from the Pseudomonadaceae family, an observation also made in previous works (Varela et al., 2018). This family is known to include some genera with great functional diversity, including some taxa that produce resistance biofilms or metabolites that inhibit *Bd* growth (Becker et al., 2015; Wei & Ma, 2013). This is in line with the identification of a high proportion of putatively *Bd*-inhibitory skin bacteria in more basic waters, being in accordance with previous studies that also suggested that populations naïve to *Bd* can often carry more anti-*Bd* bacteria but also more diverse skin communities (Kärvemo et al., 2018; Rebollar et al., 2016). Recent field assessments did not find any evidence of Bd presence in the studied area (Bletz et al., 2015), however, higher occurrence of anti-Bd bacteria may on the long run also turn the amphibians hosts more resistant to *Bd* infections (Woodhams et al., 2007).

CONCLUSIONS

This work is a preliminary survey on the possible effects of habitat alteration on skin-associated bacteria in two native amphibian species from Madagascar. The overall alpha and beta diversity do not seem to be affected by habitat type but more samples are needed to confirm these results. We observed a sharp decrease in alpha diversity in individuals from the species commonly found in rice fields when they were sampled from a site associated with a more basic water pH. Our study suggests that the effects might be more evident in terms of functional diversity, such as the proportion of anti-*Bd* taxa, therefore potentially influencing host defenses. A more detailed survey will be needed to understand if the effects on bacteria diversity and functional diversity would indeed change consistently in anthropized habitat and if those pose a risk to amphibians' native populations.

AUTHOR CONTRIBUTIONS

AC and KPM designed the study; KPM, DE and GTA performed fieldwork; BS did the laboratory work and performed the data analysis. BS and AC wrote the first version of the manuscript; all authors revised and contributed to write the paper.

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CHAPTER III

Characterization of the microbiome of the invasive Asian toad in Madagascar across the expansion range and comparison with a native co-occurring species

Paper submitted

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ABSTRACT

Biological invasions are on the rise, each invader carrying a plethora of associated microbes. These microbes play important, yet poorly understood, ecological roles that can include assisting the hosts in colonization and adaptation processes or as possible pathogens. Understanding how these communities change in an invasion scenario may help to understand the host's resilience and adaptability. The Asian toad, *Duttaphrynus melanostictus* is an invasive amphibian from Asia, which has recently been established in Madagascar and is expected to become a threat to the native ecosystems. We characterized the skin and gut bacterial communities of the toad in Toamasina (Eastern Madagascar), and compared them to those of a co-occurring native frog species, *Ptychadena mascareniensis,* at three sites where the toad arrived in different years. Microbial composition did not vary among sites, showing that the toad keeps a stable community across its expansion but significant differences were observed between these two amphibians. The invasive toad carried richer and more diverse communities with greater differences at lower taxonomic levels. The invasive toad also harboured a high percentage of total unique taxa (skin: 80%; gut: 52%). These differences may reflect the combination of multiple host-associated factors including microhabitat selection, skin features and dietary preferences. This study is the first to characterize the microbiome of an invasive amphibian species that only very recently established in the new territory while comparing with a native species that has been there for several years, and intends to set a basis for future research on the toad symbiotic communities associated with its occupation in Madagascar.

Keywords *Duttaphrynus melanostictus, Ptychadena mascareniensis,* invasive species, Toamasina, Madagascar, 16s rRNA sequencing, gut bacteria, skin bacteria

INTRODUCTION

Biological invasions can cause dramatic biodiversity loss (Chornesky & Randall, 2003; Enserink, 1999; Penk et al., 2016); with climate change, habitat alterations and direct anthropogenic translocation being the main factors facilitating the worldwide spread of alien, invasive species (Alpert et al., 2000; Crooks et al., 2011; Stachowicz et al., 2002; Walther et al., 2009). Although alien invasive species do not always have detrimental effects (Schlaepfer et al., 2011), their devastating potential can be stronger in fragile island ecosystems. Here, they often out-compete or predate on local species, interfering with

trophic networks and ultimately altering natural ecosystem' functions and balance (Lowe et al., 2000; Pitt et al., 2005). Among amphibians, notable invasive species include *Rhinella marina*, *Eleutherodactylus coqui* and *Lithobates catesbeianus* (Beard & Pitt, 2005; Shine, 2010; Snow & Witmer, 2010) of which the former is especially notorious for its negative effects in its invasive range in Australia. Among the many impacts that an invasive species can have on the native ecosystems, the introduction and spread of pathogenic fungi and viruses is also emerging as an important factor that may contribute to the global amphibian population decline (Miaud et al., 2016).

Microbiome research with high-throughput DNA sequencing techniques has enabled a better understanding of how host-associated microbiomes vary across host species, age, sex and habitats, and how their composition and diversity is influenced by host species-specific and habitat-dependent factors (Bletz et al., 2017a; Mckenzie et al., 2011b; Tiede et al., 2017). Symbiotic microbial communities likely inhabit all multicellular organisms and play an important role in the ecology, physiology, behavior and health of their hosts (Abdallah et al., 2017; Dethlefsen et al., 2007; Grice & Segre, 2012; Lester et al., 2017). The skin microbiome can influence host's ability to cope with environmental and habitat conditions and mediate immune responses (Grice, 2014; Jani & Briggs, 2018; Rebollar et al., 2016; Sanford & Gallo, 2013; Xavier et al., 2019), while gut-associated microbes can aid in food digestion, energy harvesting, development or immunity (Heijitx et al., 2011; Tuddenham & Sears, 2015; P. Turnbaugh et al., 2006). Microbiome has been proposed to affect the host's capacity for colonization, adaptation, and boosting the immune system (Cheng et al., 2018; Gribben et al., 2017; Rout et al., 2013). For example, interactions between invasive plants and associated microbes were found to suppress the rhizosphere microbes and other beneficial symbionts in native plants (Coats & Rumpho, 2014); on the contrary, some fungal symbionts were found to increase survival of their insect hosts when these were exposed to pathogens (Konrad et al., 2014). In amphibians, only a few recent studies have evaluated the microbial communities in invasive species (Abarca et al., 2018; Christian et al., 2018; Kueneman et al., 2019).

Madagascar is one of the most celebrated biodiversity hotspots (Ganzhorn et al., 2001), known not only for the high degree of endemism but also for the ongoing loss of its original primary vegetation. Amphibian diversity in Madagascar is exceptionally high (Brown et al., 2016; Perl et al., 2014; Vieites et al., 2009) and severely threatened by habitat loss and human exploitation (Harper et al., 2007). Invasive species and pathogens in Madagascar

are emerging as a new conservation concern since they may push native species further towards extinction (Bletz et al., 2015; Goodman et al., 2017; Kull et al., 2014). A naturalized population of the Asian toad *Duttaphrynus melanostictus* was reported in Madagascar in 2014, and has since become a major conservation concern (Andreone et al., 2014; Crottini et al., 2014; Kolby, 2014). Originally from Asia, it is estimated to have been present in Madagascar since 2010, being first reported near the seaport city of Toamasina, on Madagascar's eastern coast in 2014 [Fig 1; 49]. The Asian toad is believed to have arrived from Cambodia or Vietnam (Vences et al., 2017), possibly in shipping containers. At present, it occurs mainly in urban and rural lands with mixed *Eucalyptus* forests, where native amphibian communities are highly impoverished. However, it is rapidly expanding (Licata et al., 2019) and it is feared that it may soon reach areas known to host richer amphibian communities such as Betampona Strict Nature Reserve and Ivoloina (Crottini et al., 2014; Rosa et al., 2012). *D. melanostictus* is known to have a high invasive potential (Reilly et al., 2017). Although with low incidence, predation of smaller herpetofauna has been observed in other invasive populations of the toad (Döring et al., 2017), and there is a concern that in Madagascar it may start to predate on native amphibian species. The Asian toad is also known to produce highly poisonous skin toxins that are likely to negatively affect the vast majority of potential native predators (Marshall et al., 2018). Skin secretions isolated from *D. melanostictus* individuals collected in its native range seem to contain potent antimicrobial agents and important pharmacological compounds (Garg et al., 2007) that may increase disease resistance, making this invasive amphibian species a particularly interesting candidate for microbiome studies in invasive scenarios.

Here, we provide the first assessment of skin and gut bacterial communities of the invasive Asian toad *D. melanostictus* population after its recent introduction to Madagascar and we compare its microbiome with the one hosted by a co-occurring native species, the Mascarene grass frog, *Ptychadena mascareniensis*, across its expansion range over the last few years. We hypothesize that the invasive species will present richer and more diverse bacterial assemblages due to its We hypothesize that the invasive species will present richer and more diverse bacterial assemblages due to its distinctive ecological and behavioral preferences and because it is expected to comprised a mix of microbes from the toad's native and introduced habitats. We expected that across sites the toad may have different microbial assemblages. We explore the correlation between the toad's microbial diversity and its high colonization and adaptation capacity, using as proxy measures of bacterial

species richness, diversity and functional inference that may confer disease resistance or enhance wider diet range in different habitats.

METHODS

Sampling

Sampling was performed between September 20th and 24th, 2016. We collected 16 individuals (8 males and 8 females) of the invasive Asian toad *Duttaphrynus melanostictus* and 3-4 individuals of the native and co-occurring species *Ptychadena mascareniensis*, at three sites across the invaded area around Toamasina (eastern Madagascar) (Fig. 1); the low number of collected individuals of *P. mascareniensis* resulted from the low population density at the time. The selected sites are highly anthropogenically transformed areas and are typically occupied by *D. melanostictus* (Licata et al., 2019). Selection of sampling sites was based on the distribution data available for *D. melanostictus* at the time (retrieved from Moore *et al*. (Moore et al., 2015) and based on field observations carried out by the staff of Madagascar Fauna and Flora Group). We aimed to include one sampling site where the toad had only recently established (Fig. 1): Site 1 (green), possible point where the toad first colonized in 2010; Site 2 (orange), site where the toad was observed during a survey performed in 2015; Site 3 (blue), site where in 2014 the toad had not been detected yet; polygon corresponds to the identified distribution area of the toad during the survey of 2014 (Moore et al., 2015). Each specimen was collected with new nitrile gloves, measured (snout-vent length and weight), and kept in individual sterile plastic bags until sampling. Each specimen was rinsed with sterile water to remove debris and transient microbes, and swabbed 10 times on the ventral side and five times on each thigh and foot using one sterile swab (MW113, Medical Wire Equipment & Co. Ltd., Corsham, United Kingdom). To characterize the gut bacterial communities, four individuals from each site per species were euthanized with a solution of Tricaine Methanesulfonate (MS-222, Sigma-Aldrich), and the gut was collected by dissection. Gut samples per site and per species were pooled (e.g., the pooled sample of *D. melanostictus* from each site included 2 guts from male individuals and 2 guts from female individuals; the pooled sample of *P. mascareniensis* individuals from each site included 4 guts of juveniles (sex undetermined)) and stored in RNA later, kept in liquid nitrogen during the sampling period, transferred to cool conditions during the export from Madagascar, and stored at -80ºC upon their arrival to Europe. Swabs were kept dry in

cool conditions during the sampling period and the export from Madagascar, and transferred to -20ºC upon their arrival in Europe.

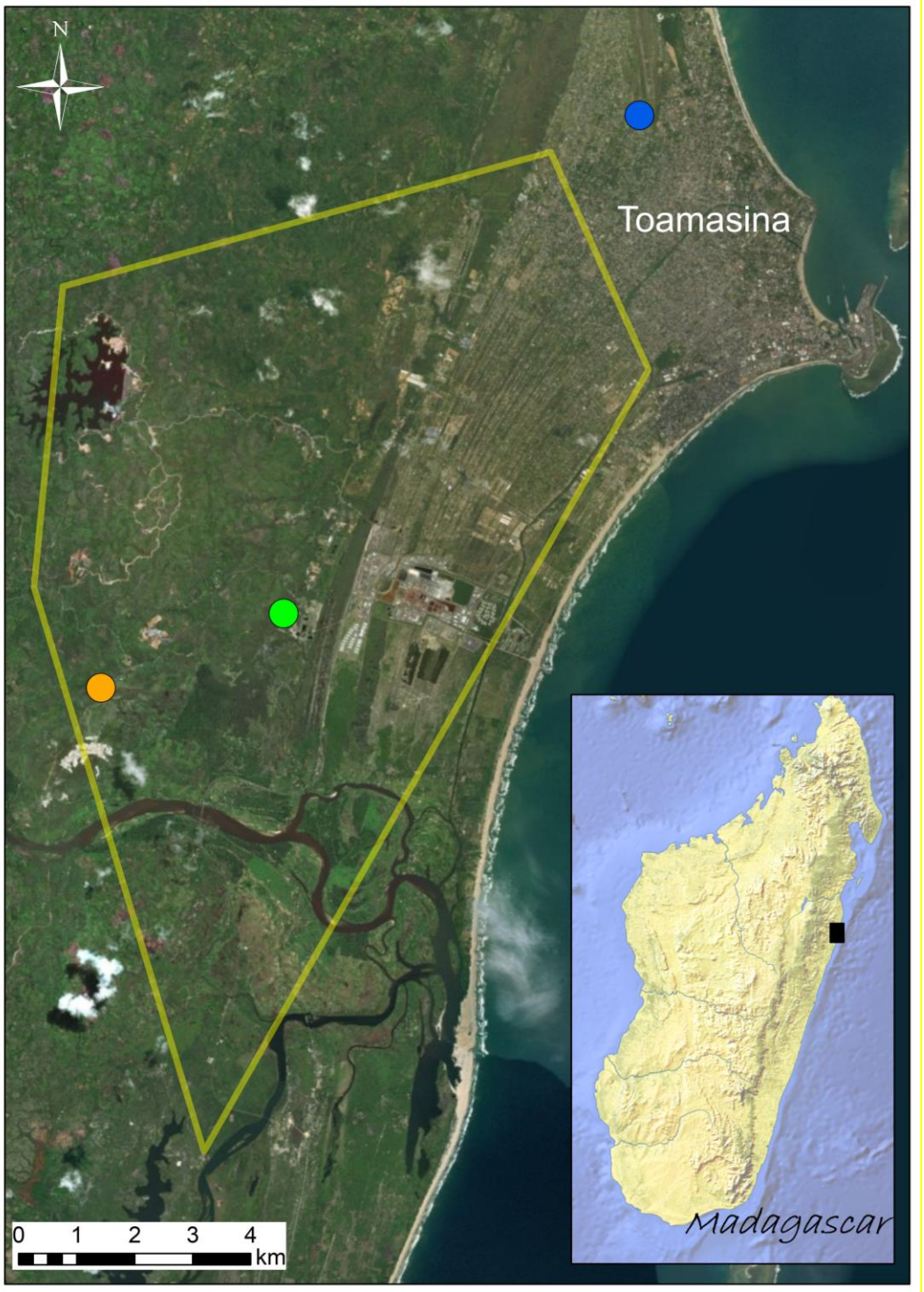

Figure 3. 1 Distribution of the sampling sites (Site 1 = green; Site 2 = orange; Site 3 = blue) visited in September 2016. Known distribution area of the invasive population of *Duttaphrynus melanostictus* in Toamasina in late 2014 (yellow polygon; modified from Moore et al., 2015.

DNA extraction, amplification and sequencing

DNA was extracted following a modified Qiagen DNeasy Blood & Tissue Kit protocol (Hilden, Germany) with an initial lysozyme incubation step at 37ºC to break up cell walls of Gram-positive bacteria. The V4 region of the bacterial 16S rRNA gene was amplified with barcode primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (10µM). Amplification was performed in duplicate with an overall volume of 12.5 µl including 0.2 µl of Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, Ma, USA), 0.25 µl of each primer (10 µM), 0.25 µl of dNTPs, 2.5 µl of buffer, 8.1 µl of H_2O and 1 µl of template DNA. The amplification protocol consisted of an initial denaturation step at 98ºC for 1 min, followed by 30 cycles of denaturation at 98ºC for 10 s, annealing at 55ºC for 30 s and elongation at 72ºC for 30 s, with a final extension at 72ºC for 5 min. PCR products of the two replicates were pooled in a total volume of 25 µl and was visualized on 1% agarose gel. All samples were pooled together according with band size and the final pooled sample was run in a 1% agarose gel and purified with QIAQuick Gel Extraction Kit (Qiagen, Hilden, Germany). Samples were sequenced using paired-end 2 x 250 v2 chemistry on an Illumina MiSeq sequencing platform using a dual-index approach (Kozich et al., 2013). Raw sequencing data are available in the NCBI short read database (Bioproject PRJNA667830).

Sequence processing

Sequences were processed in Quantitative Insights into Microbial Ecology (QIIME v1.9.1) (Caporaso et al., 2010). Due to the typical lower quality of reverse reads (Kwon et al., 2013), only the forward reads were filtered following the criteria: absence of Ns within the sequence, absence of barcode errors, and exclusion of reads containing at least three consecutive low-quality nucleotides. Sequences were clustered into sub-operational taxonomic units (sOTUs, hereafter called OTUs) following the deblur workflow (https://github.com/biocore/deblur) (Amir et al., 2017). Sequences were trimmed to 150 bp and OTUs with less than 10 reads were excluded. The resulting OTUs were then assigned to a taxonomic group using the Greengenes 13.8 reference database (May 2013 release; http://greengenes.lbl.gov/). Non-bacterial taxa (e.g.: mitochondria and chloroplasts) were removed from the dataset. All OTUs with less than 0.001% of the total reads of all analyzed samples were excluded (Bokulich et al., 2013). PyNAST (Caporaso et al., 2010) was used to align the OTUs sequences and a phylogenetic tree was built with FastTree (Price et al., 2010). The final datasets included a total of 37 samples with 1,617 OTUs for the skin bacteria

dataset and a total of 6 pooled samples (total of 12 individuals per species with 4 individuals pooled per site) with 701 OTUs for the gut bacteria dataset (Table S1).

Statistical analysis

Diversity indices and statistical analysis were performed using QIIME v1.9.1 and R v3.4.4 (R Core Team, 2016). Data was organized into three datasets and each were rarified to a specific number of reads per sample (Tables S1, S2). The exception was Dataset A, where the samples were rarefied at two different levels: the lower rarefaction level (1,455 reads/sample) was used for all the analysis; the higher level (4,000 reads/sample) was used only to calculate alpha diversity indices to allow further comparison with previous works with Malagasy amphibians. DATASET A included a total of 37 skin swabs from both species from all sites and was used to assess the effects of host and site on skin bacteria; DATASET B included 26 skin swabs from *D. melanostictus* from all sites and was used to assess the effect of the sex on skin bacteria*;* DATASET C included four pooled gut samples per species per site and was used to assess the effects of host species on the gut bacteria (Tables S1, S2 for more details).

Alpha diversity metrics were calculated for each sample and included species richness measured as number of observed OTUs (OTU Richness) and Chao1 diversity index; and diversity measured using Shannon diversity index and Faith's phylogenetic distance (PD). Significant differences between alpha indices were assessed using ANOVA (aov, stats package, DATASET A: two-way ANOVA, factors "species" and "site"; DATASET B: one-way ANOVA, factor "sex"; DATASET C: one-way ANOVA, factor "species"). Dissimilarity matrices were calculated using Weighted and Unweighted Unifrac distances (Lozupone & Knight, 2005) and visualized using a non-metric multidimensional scaling (NMDS, phyloseq package). Differences in the bacterial community structure (Beta Diversity) were analyzed with PERMANOVA (Adonis, vegan package, 999 permutations (Oksanen et al., 2017)). Community composition was visualized with bar plots including the 10 most abundant taxa in each category (phylum, family and genus) after transforming the counts into relative abundances. Total shared and unique OTUs for each species were represented as Venn diagrams for all groups using the collapsed biom tables retrieved from QIIME1. Since no significant differences were found between sites, the subsequent analysis was performed with individuals from the three sites grouped together. Linear Discriminant Analysis Effect Size (LEfSe) method (LDA score > 3.0, α=0.05) (Segata et al., 2011) was used to determine OTUs responsible for the observed differences in the skin and gut communities between

species (DATASETs A and C) and sexes (DATASET B). Functional predictions of the skin and gut bacterial communities were extrapolated from the 16S data using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013). The OTUs were assigned to the Greengenes v13.5 database using the 97% similarity with the closed OTU-picking strategy. On the assigned OTU table, a normalization of the copy numbers of each OTU was performed. Subsequently, the metagenome of each sample was predicted, and a functional categorization with respective abundances (following the Kyoto Encyclopedia of Genes and Genomes – KEGG – Orthology database) performed, using level 2 KEGG Orthologs (KO). Pathways with less than 10 counts were removed and abundances were rarefied. Both LEfSe and PICRUSt analysis were run on the Galaxy Web platform (http://huttenhower.sph.harvard.edu/galaxy). Significant differences between host species were assessed using the Kruskal-Wallis test (K-W) in QIIME. All skin bacterial OTUs from DATASETs A and B were mapped against the published database of antifungal amphibian skin bacterial isolates (Douglas C. Woodhams et al., 2015) - this database includes isolates that are likely able to inhibit the growth of the amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*) - and the proportions of both *Bd*-inhibitory and *Bd*-enhancing OTUs were calculated.

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Ministère de l'Environnement et du Développement Durable provide the research permits for Collection: N°226/16/MEEF/SG/DGF/DSAP/SCB.Re of September 19th, 2016; Transport: N°1679-16/MEEF/SG/DGF/DREEF.ATS/SREco and N°1680-16/MEEF/SG/DGF/DREEF.ATS/SREco of September 24th, 2016; Export: N°284N-EA10/MG16 of October 5th.

RESULTS

Dataset A - Comparison of the skin microbiome of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis* across the expansion range

Host species had a significant effect on alpha diversity indices of the cutaneous microbiome (Fig. 3.2A): *D. melanostictus* showed significantly higher values for bacterial richness (ANOVA, $F=33.15$, $p<0.001$), phylogenetic diversity (ANOVA, $F=40.66$, $p<0.001$), Chao1

diversity (ANOVA, F=29.64, p<0.001) and Shannon diversity (ANOVA, F=7.289, p<0.05) than the native *P. mascareniensis* (Fig. 2A; SM Table S3). Site did not have an effect on alpha diversity (ANOVA, OTUs: F=0.505, p=0.608; PD: F=1.830, p=0.180; Chao1: F=1.274, p=0.296; Shannon: F=1.074, p=0.36, SM Fig. S1), and the interaction between species and site was not statistically significant (ANOVA, OTUs: F=0.729, p=0.49; PD: F=1.078, p=0.35; Chao1: F=0.334, p=0.72; Shannon: F=0.783, p=0.47). However, a trend was observed with *D. melanostictus* showing an increase in all alpha indices across sites while *P. mascareniensis* showed an irregular pattern.

Beta diversity significantly differed between host species when measured by both weighted Unifrac (Fig. 3.2B, PERMANOVA: Pseudo-$F_{(1,36)}$=4.896, R^2=0.118, p=0.002) and unweighted Unifrac metrics (Fig. 3.2B, PERMANOVA: Pseudo-$F_{(1,36)}$=6.565, R^2=0.156, p<0.001); but did not differ across sites (Fig. 3.2B, PERMANOVA: weighted Pseudo-$F_{(2,36)}$=1.138, R^2=0.055, p=0.317; unweighted Pseudo-$F_{(2,36)}$=1.036, R^2=0.049, p=0.361). Similarly, the interaction of species and site did not affect beta diversity (Fig. 3.2B, PERMANOVA: weighted Pseudo-$F_{(2,36)}$=1.664, R^2=0.080, p=0.057; unweighted Pseudo-$F_{(2,36)}$=1.217, R^2=0.058, p=0.145).

The skin bacterial communities from the two species were mainly composed by the same phyla (Actinobacteria, Bacteroidetes and Proteobacteria) but with several differences in relative abundances at lower taxonomic levels (family and genus; Fig. 3.2C). The *D. melanostictus* skin community had higher abundances of the families Alteromonadaceae, Comamonadaceae, Moraxellaceae and Sphingobacteriacae while the *P. mascareniensis* skin community had higher abundances of Enterobacteriaceae, Moraxellacaeae (only at Site 3), Pseudomonadaceae and Xanthomonadaceae. Notably, *P. mascareniensis* had a higher abundance of bacteria of the genus *Pseudomonas* while *D. melanostictus* had *Cellvibrio* as the most abundant genus. Across sites, the differences observed between host species were concordant. Within species, the patterns varied: *D. melanostictus* skin bacterial communities were more stable across sites and *P. mascareniensis* showed more variability in groups abundance (Fig. 3.2C).

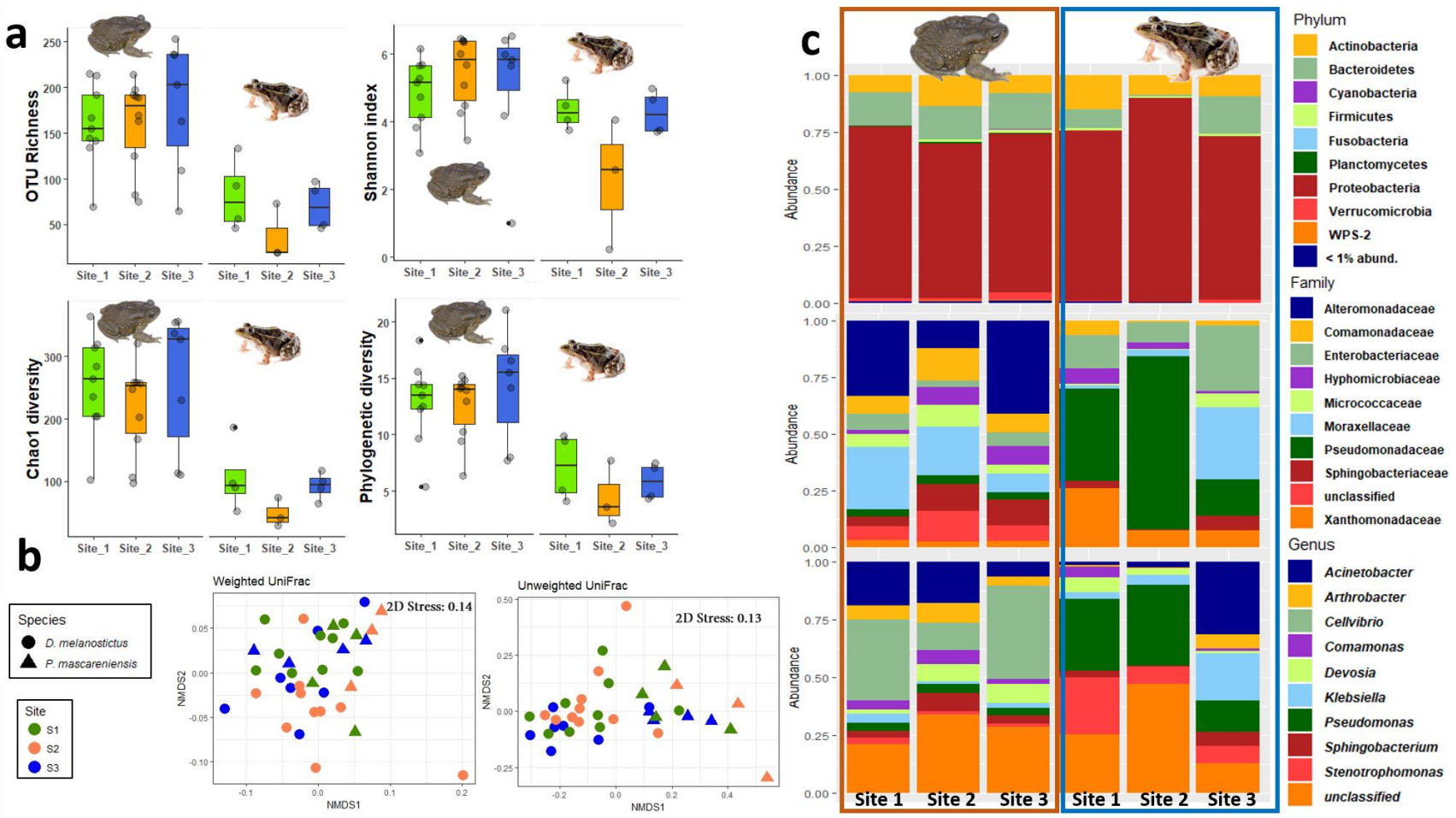

Figure 3. 2. Skin bacterial diversity and composition of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis* across species: (a) Alpha Diversity metrics were all significantly different between host species ($p<0.05$) but not across sites ($p>0.05$); b) Skin bacterial structure of *D. melanostictus* (circles) and *P. mascareniensis* (triangles) across the three sampling sites using a non-metric multidimensional scaling (NMDS) ordination of Weighted and Unweighted Unifrac distances, c) Composition of the skin bacterial communities including the 10 most abundant Phyla, Families and Genera in *D. melanostictus* (left panel) and *P. mascareniensis* (right panel) across the three sampling sites.

In total, the toad had more than 1,000 unique OTUs (equivalent to 80% of total number of OTUs) and shared only 238 (15%) with *P. mascareniensis*, while the latter had only 5% unique OTUs (Fig. S1A). The percentage of shared OTUs between the species was similar at sites 1 and 3 and lower at site 2 (Fig. S1C). The lower percentage was coupled with higher number of unique OTUs found in the toad (Fig. S1C). Toad individuals across sites shared between 30-40% of OTUs, while the percentage of unique OTUs found at each site were around 20%; individuals from sites 1 and 2 shared more OTUs than in comparison with the number of shared OTUs between each of the first two sites with site 3 (Fig. S2B). *P. mascareniensis* had similar trends, with individuals sharing a higher number of OTUs compared with the unique OTUs found at each site. However, the percentage of OTUs found at the three sites was only 9% (Fig. S2B).

LEfSe analysis revealed 41 OTUs that were differently abundant in the two host species including 14 OTUs that exhibited higher relative abundance in *P. mascareniensis* and 27 in *D. melanostictus* (Fig. 3.3). Specifically, *P. mascareniensis* only had OTUs from the phylum Proteobacteria and a single OTU from the phylum Firmicutes, while *D. melanostictus* had OTUs from the Actinobacteria, Proteobacteria, Bacteroidetes and Verrucomicrobia phyla. In *P. mascareniensis*, all differentially abundant OTUs were included within the class Gammaproteobacteria with the exception of one Alphaproteobacteria OTU. In the case of *D. melanostictus*, differentially abundant bacteria belonged to several classes and families within different phyla (Fig. 3.3).

A total of 39 KEGG pathways (Level 2) were predicted for the two amphibians' skin microbiomes, of which 18 exhibited significantly different relative abundance between species (Table S3). From these, *D. melanostictus* had 11 enriched functional groups including cell growth and death, transport and catabolism, biosynthesis of secondary metabolites, energy and lipid metabolism, xenobiotics biodegradation and environmental adaptation. *P. mascareniensis* had 7 enriched functional groups including membrane transport, infectious diseases, cellular processes and signaling, among others.

The two amphibians had significant differences in the proportion of putatively *Bd*-inhibitory but not in *Bd*-enhancing skin bacteria (K-W test, $\chi2=11.5$, $p<0.001$ and $\chi2=3.10$, $p=0.078$ respectively) with *P. mascareniensis* carrying higher proportions of *Bd*-inhibitory bacteria (Fig. 3.4).

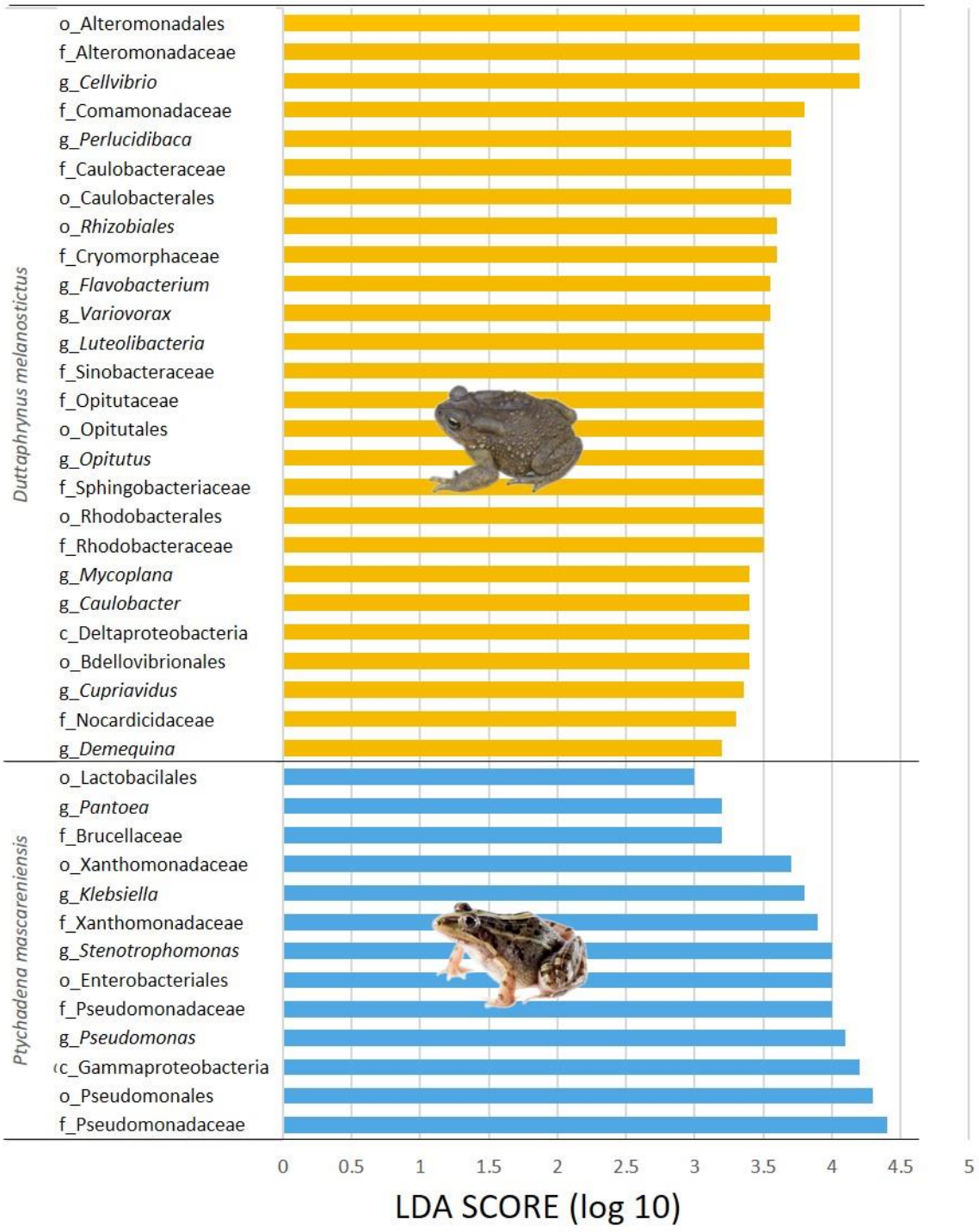

Figure 3. 3. Differentially abundant skin OTUs occurring in *Duttaphrynus melanostictus* (yellow bars) and *Ptychadena mascareniensis* (blue bars) – LDA scores of detected OTUs in LEfSe analysis.

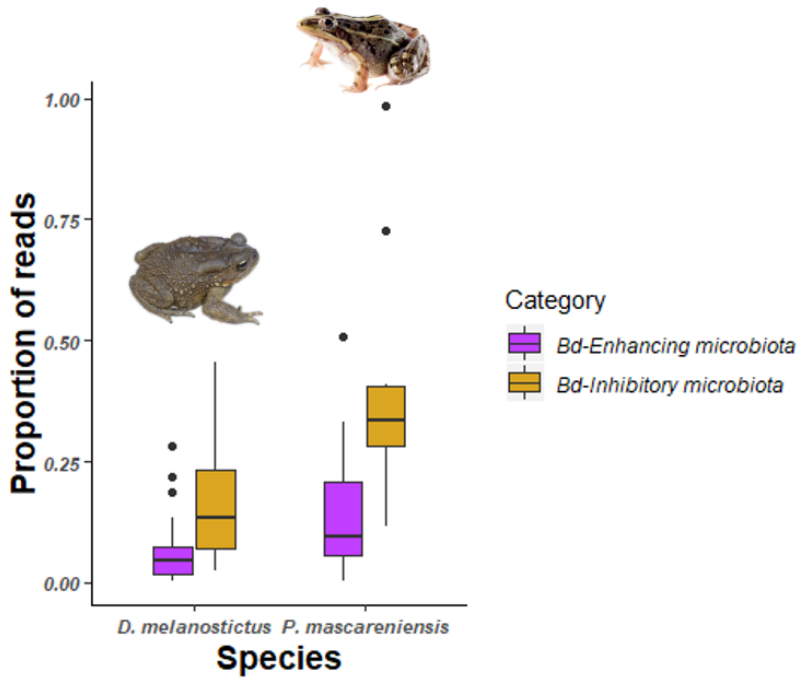

Figure 3. 4. Proportions of reads of *Bd*-Inhibitory (yellow) and *Bd*-Enhancing (purple) skin OTUs from *Duttaphrynus melanostictus* and *Ptychadena mascareniensis* compared to the Antifungal Isolates Database [69].

Dataset B - Comparison of the skin bacterial community of males and females of *Duttaphrynus melanostictus*

No significant differences between sexes or sites were found in terms of alpha diversity (except for Shannon index where sex was significant (p=0.04), Fig. 3.5A). Beta diversity showed that only sex was significant and only when using weighted Unifrac distances (PERMANOVA: Sex, Pseudo-F=2.35, R^2=0.09, p=0.02, Fig. 3.5B). In the Unweighted Unifrac matrix, none of the factors was significant (PERMANOVA: Sex, Site, p>0.05, Fig. 3.5B). Males from the three sites had higher abundances of Sphingobacteriacae and a high rate of unidentified taxa when compared with females (Fig. 3.5C). At the genus level, both sex and site seemed to influence the abundance level of the most common taxa (*Arthrobacter, Cellvibrio, Devosia*) but without a clear pattern. Once again, males had a higher abundance of unidentified genus than females (Fig. 3.5C).

Males had the double number of unique OTUs than females when samples from the three sites were grouped (Fig. S2), although it can also be linked with the higher number of samples. Functional and Woodhams analysis showed minor differences while no differently abundant taxa were found in either sex using LEfSe analysis (Fig. S3, Table S4).

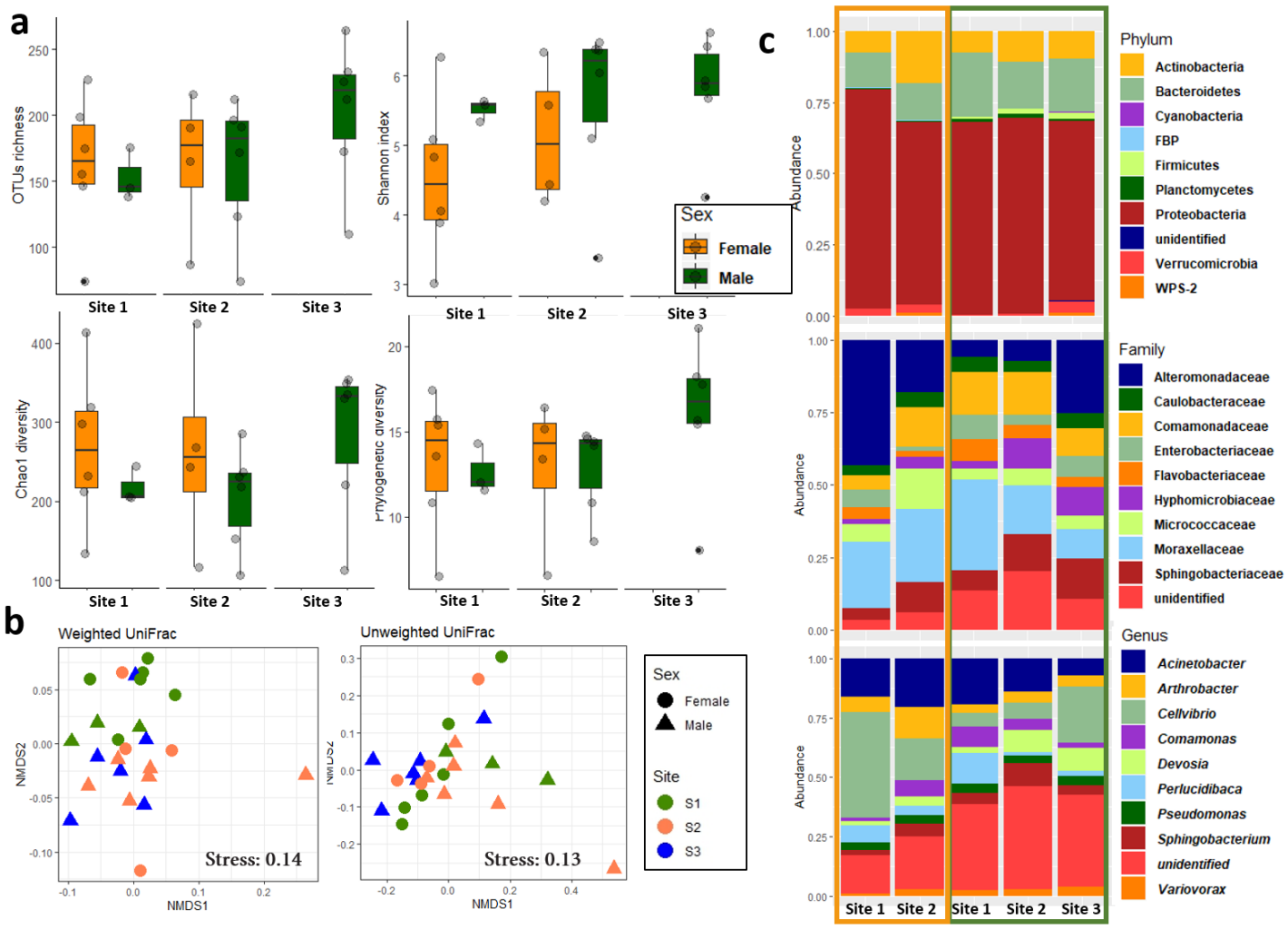

Figure 3. 5. Skin bacterial diversity and composition of males and females of *Duttaphrynus melanostictus* across sites: (a) Alpha Diversity metrics were not significantly different between sexes (p>0.05) or across sites (p>0.05); b) Skin bacterial community structure of females (circles) and males (triangles) across the three sites using a non-metric multidimensional scaling (NMDS) ordination of Weighted and Unweighted Unifrac distances, c) Composition of the skin bacterial community including the 10 most abundant Phyla, Families and Genera in females (left orange panel) and males (right green panel) across the three sampling sites.

Dataset C - Comparison of gut bacterial communities of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis*

In dataset C, only one pooled sample (with four individuals each) per site and species was obtained, thus all the statistics were performed to compare only the effect of the host species (Fig. 3.6A). The complete plots with separated sites are available in supplementary material (Fig. S4A). The gut communities did not present significant differences between host species for any of the indices (ANOVA, OTUs: $F_{1,5}$=3.46, p=0.14; Chao1$_{1,5}$: F=2.08, p=0.22; PD: $F_{1,5}$=3.46, p=0.14; shannon: $F_{1,5}$=1.56, p=0.28) although a trend for an increase in bacterial richness and diversity can be observed in *D. melanostictus* (Fig. 3.6A; SM Fig. S4A).

No significant differences in gut community composition were found between the two species using both weighted Unifrac (PERMANOVA: Pseudo-$F_{1,5}$=1.66; R^2=0.3, p=0.3) and unweighted Unifrac (PERMANOVA: Pseudo-$F_{1,5}$=1.75; R^2=0.3, p=0.1) distances (Fig. 3.6B, Fig.S4B).

The gut community of both species was dominated by three phyla (Bacteroidetes, Proteobacteria and Firmicutes) (Fig. 3.6C; Fig. S4C), with differences in the relative abundances at lower taxonomic levels. Higher abundances of the classes Bacteroidia and Clostridia classes were found in *D. melanostictus* while in *P. mascareniensis*, the classes Bacilli and Clostridia were the more abundant. At the family level, *D. melanostictus* showed a more homogeneous gut community including 10 abundant families (Fig. 3.6C), while *P. mascareniensis* had higher abundances of only two families: Clostridiaceae and Streptococcaceae. At the genus level, the gut community of *D. melanostictus* was dominated by *Bacteroidetes* and *Cetobacterium* while *P. mascareniensis* had higher abundances of *Clostridium* and *Lactococcus*.

D. melanostictus had 365 unique OTUs (52%), while *P. mascareniensis* had 256 (37%) and only 11% of the bacterial OTUs (corresponding to a total of 80 OTUs) was shared (Fig. S5).

A total of 22 OTUs were significantly more abundant in the gut of *D. melanostictus*, these included members of the three phyla (Bacteroidetes, Firmicutes, Proteobacteria) while no OTUs were enriched in *P. mascareniensis* (Fig. 3.7).

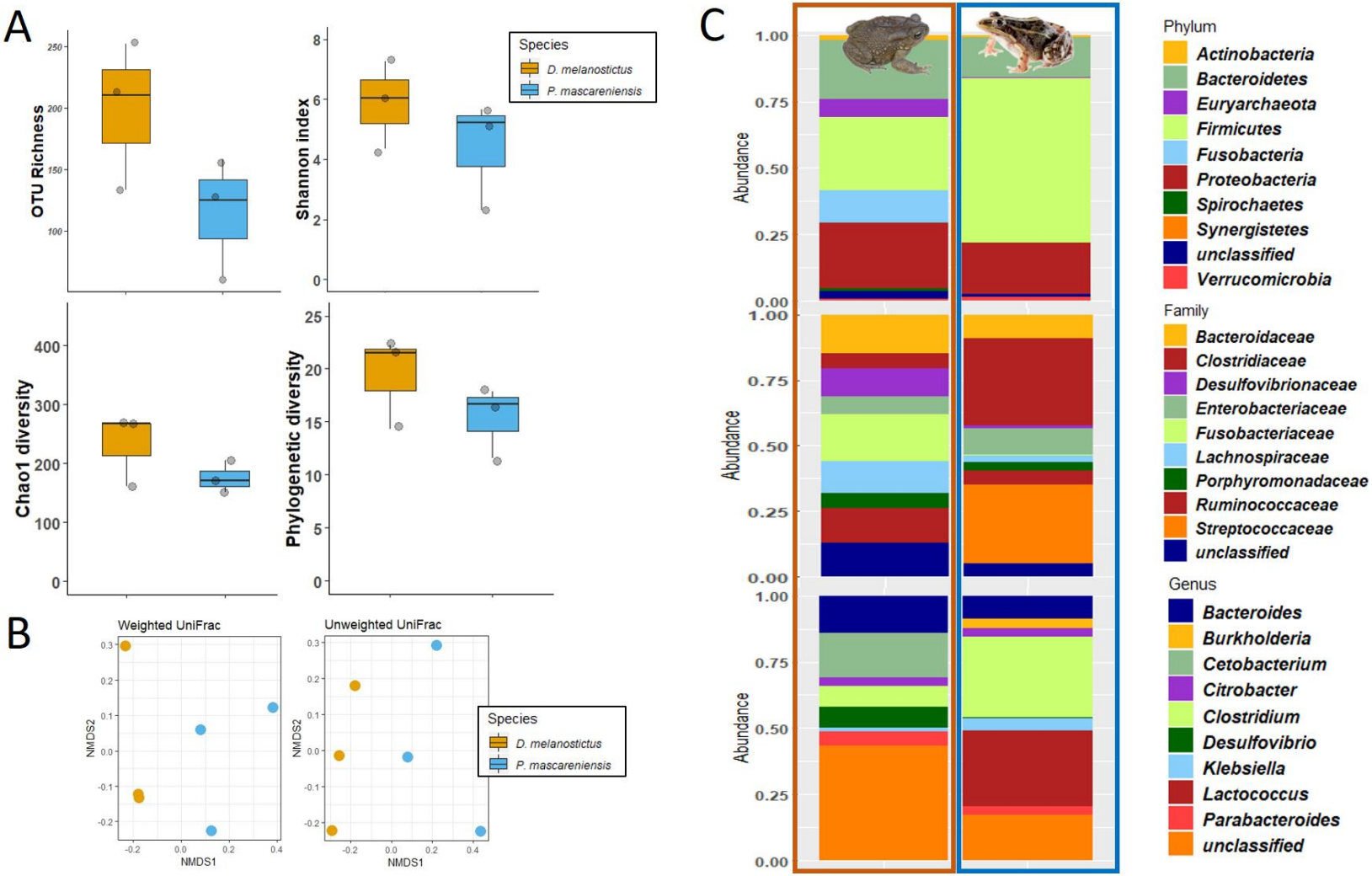

Figure 3. 6. Gut bacterial diversity and composition of *Duttaphrynus melanostictus* (yellow) and *Ptychadena mascareniensis* (blue) across species: a) Alpha Diversity metrics with no significant differences (p>0.05); b) Gut bacterial community structure of *D. melanostictus* (circles) and *P. mascareniensis* (triangles) across the three sites using a non-metric multidimensional scaling (NMDS) ordination of Weighted and Unweighted Unifrac distances, c) Composition of the gut bacterial communities including the 10 most abundant Phyla, Families and Genera in *D. melanostictus* (left panel) and *P. mascareniensis* (right panel) across the three sites.

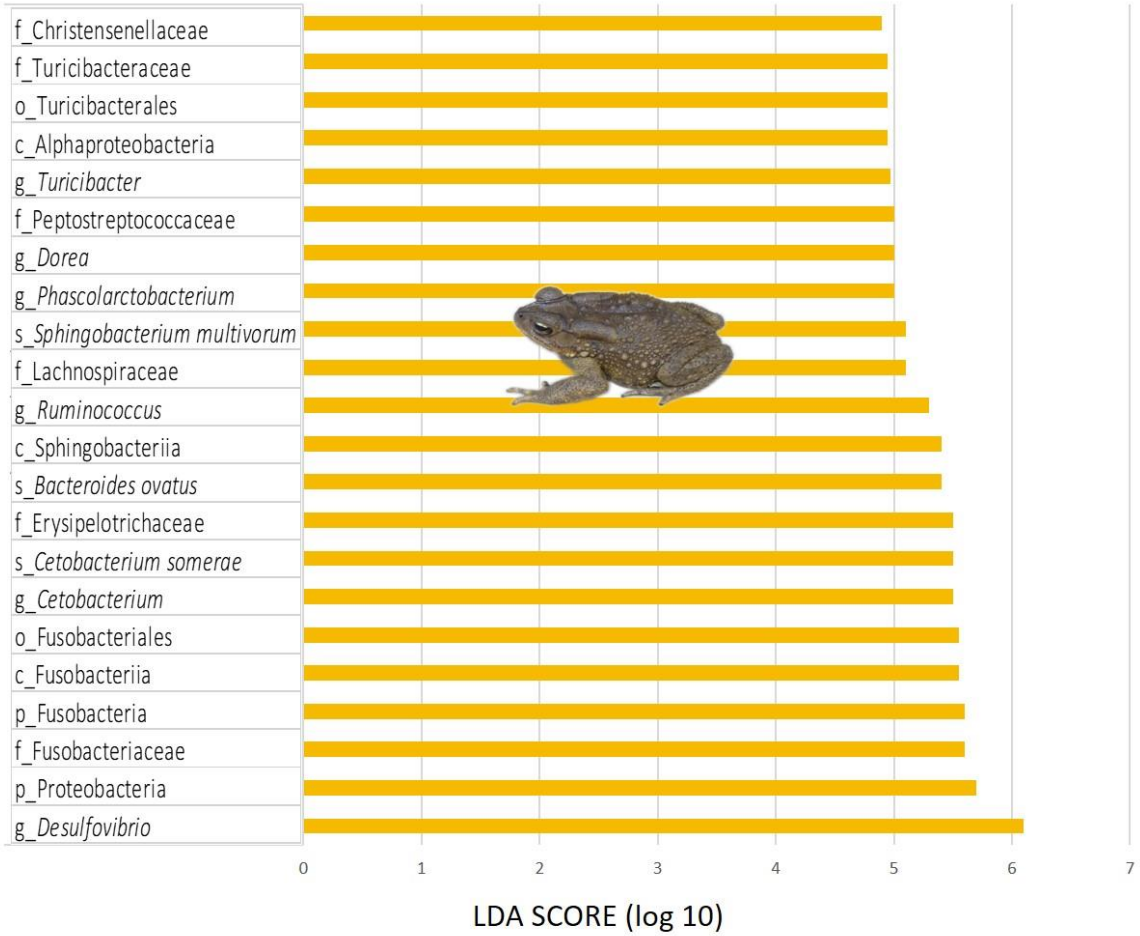

Figure 3. 7. Differentially abundant gut OTUs occurring in *Duttaphrynus melanostictus* with LDA score. No differentially abundant gut OTUs were identified in *P. mascareniensis*.

From the gut communities of the two species a total of 39 KEGG pathways (Level 2) were predicted, with both amphibians exhibiting the same functional groups. Significant differences in the abundance levels of these pathways were detected (K-W, p-value < 0.05) (Table S5). *D. melanostictus* had 4 enriched functional groups: Biosynthesis of Secondary Metabolites, Energy Metabolism, Endocrine System and Information Processing – Folding, Sorting and Degradation, while *P. mascareniensis* had only one enriched functional group associated with membrane transport (Table S5).

DISCUSSION

Our study provides the first characterization of the skin and gut microbiomes of the invasive toad *Duttaphrynus melanostictus* in its invasive range in Madagascar occurring in a highly human impacted area and includes a comparison with the co-occurring native species *Ptychadena mascareniensis*. To our knowledge, only four recent studies characterized the microbiome of an invasive amphibian: Christian *et al*. (Christian et al., 2018) found that *Rhinella marina* had the poorest and most dissimilar skin bacterial community in comparison with native amphibians in Australia, while Abarca *et al*. (Abarca et al., 2018) found, for the same species, higher skin bacterial diversity in individuals from the invaded range compared to the native range; finally, Kueneman *et al*. (Kueneman et al., 2019) found that on a global scale the skin microbiome of *Lithobates catesbeianus* was more similar to that of the native amphibians than to itself in different parts of its invasive range. A more recent study focused instead on the gut microbiome of *R. marina* while comparing the bacterial community across gut sections (Zhou et al., 2020). Differently from these studies, we investigated a very recent invasion and aimed to know how the toad microbiome change across three sites within its expansion range across the years and to address the differences when in comparison with the microbiome of a native species from the same impoverish sites. As expected, bacterial communities were strongly correlated with host identity, with the Asian toad showing higher richness and diversity. However, no significant differences were observed between sampling sites for the toad, which might be related to the still small and homogeneous area, and therefore limited environmental variability, where the toad can be found but also indicates that the toad microbiome has been stable across its expansion.

Skin bacteria diversity differs between the invasive and the native hosts but not across sites

The skin bacterial community of the two amphibian species differed in terms of richness, diversity, community structure and functional inferences. The invasive *D. melanostictus* hosted a richer community than the co-occurring native species, including most of the unique OTUs (80%). *P. mascareniensis* showed low bacterial richness in comparison with the toad, and lower than the average values found in other terrestrial or aquatic amphibians from Madagascar while the invasive toad seems to have a bacterial richness that is within the average values for terrestrial species in Madagascar (Table S6, (Bletz et al., 2017; Kueneman et al., 2019)). It should be noted however, that while previous studies included terrestrial amphibians from different habitats across Madagascar (Bletz et al., 2017), our study included only sites from Toamasina, a very homogeneous area with high anthropogenic disruption that could affect the environmental

bacterial pool and consequently some impoverishment of the bacterial communities in the Asian toad but also in *P. mascareniensis* when it is compared with other Malagasy terrestrial amphibians (Table S6).

Notably, the toad showed a much higher percentage of unique OTUs (80%) than the native species that may be related with toad skin microenvironment or ecological factors. The percentage of shared bacteria (15%) between our two species was also very low compared with previous works that compare other species (25-70%), or between aquatic and terrestrial ecomorphs or different life stages (Bletz et al., 2017; Kueneman, et al., 2014; Rebollar et al., 2016). Although differences across sites were not significant, it can be observed a trend for an increase diversity in the toad across the sites. Moreover, at site 3, the toad maintains the higher number of unique bacteria while share more OTUs with *P. mascareniensis* that has much lower number of unique bacteria. Comparing with previous studies, it also seems that *Ptychadena* has a more impoverished microbiome, maybe due to the anthropized area and this should be investigated future assessments (Bletz et al., 2017). To explain differences in bacterial composition between the two target species, skin texture may also play a major role. Tubercles in *D. melanostictus* skin may provide alternative microniches for the bacteria comparing with the smoother skin of *P. mascareniensis.* Moreover, amphibians' skin has been suggested to select and filter for specific bacteria from the surrounding environment due to the secretion of skin compounds that may block colonization by some taxa and favor others, and this selection could differ among host species (Flechas et al., 2019; Walke et al., 2014a). Terrestrial amphibian species (like the Asian toad) are expected to have richer skin communities than aquatic or arboreal amphibians (Bletz et al., 2017; De Assis et al., 2017; Kueneman et al., 2019; Walke et al., 2014a) partly because the soil usually harbors a richer bacterial pool than aquatic systems and the habitat is known to greatly influence amphibians' skin communities. Although both species were found in the highly anthropized areas in Toamasina, *P. mascareniensis* were mostly found in the grass and often close to small water-bodies, while toads were conspicuous within villages, sometimes near domestic animals or anthropogenic waste.

So far, no studies have assessed the role of bacterial communities in the adaptation of amphibians to novel habitats. However, in other systems, more diverse microbiomes have been linked to higher host fitness, as, for instance, pathogen resistance in wheat (Matos et al., 2005), or defense against chemical compounds in beetles (Cheng et al., 2018). In amphibians, richer microbiomes have been linked to a higher resistance to pathogens (Becker & Harris, 2010; Harrison et al., 2017) and a richer microbiome could conceivably aid in the colonization of novel habitats.

Bacterial taxa associated with disease resistance were among the most abundant groups in both hosts but with specific differences in taxa identity and abundances. *P. mascareniensis* seems to carry a more diverse bacterial community with antifungal properties while the toad carried more OTUs from the Comamonadaceae family that contains taxa used in probiotic assays (Becker et al., 2015). The toad also had very low abundance of *Pseudomonas*. This genus is ubiquitous in the environment (soil, water), plants and other organisms and is linked to resistance to pathogens like *Bd* (Becker et al., 2015). *Pseudomonas* was highly prevalent in *P. mascareniensis* and is usually abundant in amphibians from tropical regions (Bletz et al., 2017). Notably, the low abundance of *Pseudomonas* found in the toad agrees with the pattern found in invasive populations of *R. marina*: lower in invasive populations compared with native ones (Abarca et al., 2018), and lower in comparison with co-occurring native amphibians from Australia (Christian et al., 2018) and further analysis of this similarity should be applied.

The functional redundancy here observed was congruent with previous studies (Bletz et al., 2016; Huang et al., 2018), demonstrating that different microbiome assemblages from different hosts can succeed in the same environment and are probably more associated with host identity. From the host's perspective this is crucial since it means that it maintains functional stable microbial community despite carrying different bacterial assemblages. The functional category of xenobiotics biodegradation and metabolism that was enriched in *D. melanostictus* could be related with a high capacity to cope with environmental alteration and anthropogenic stress which would be the case in Toamasina, and therefore higher adaptability or resilience to highly impacted or new habitats (Claus et al., 2016).

In a recent study with *R. marina* collected near its invasion front and where chytridiomycosis is absent, it was observed that individuals had lower *Bd*-inhibitory bacteria if compared with areas where *Bd* was present, highlighting the hypothesis that these bacteria are selected when the pathogen is present (Weitzman et al., 2019). In the species analyzed here, the lower proportion of bacteria with putative *Bd*-inhibiting functions in *D. melanostictus* (in comparison with *P. mascareniensis*) may be related with its occurrence is Toamasina where *Bd* has not yet been detected (Bletz et al., 2015). However, *D. melanostictus* have been showed to have a very high *Bd* prevalence (43%) in its native areas in India (Thorpe et al., 2018) which may be also be linked to the low proportion if *Bd*-inhibitory bacteria. A screen of the microbiome diversity of the Asian toad from its native areas and where *Bd* has been detected could give new insights about microbiome patterns.

Gut bacteria show a trend to differentiation between the two species

Similarly, to the skin communities, the Asian toad hosted a bacterial community characterized by higher number of unique OTUs and higher richness values (although the low sample size did not allow for a robust statistical analysis). Although both species are generalist feeders (Döring et al., 2017; Fatroandrianjafinonjasolomiovazo et al., 2011), *P. mascareniensis* feeds mainly on arthropods while the Asian toad diet includes other invertebrates and occasionally also small vertebrates (e.g. worm snakes) (Hahn, 1976; O'Shea et al., 2013). A larger body size probably allows the consumption of larger and more diverse prey and the microhabitat type (soil, water, leaves) may also hold different invertebrate groups influencing the potential prey availability for the two species. A richer bacterial community has been related with richer diets probably aiding the host in the digestion and metabolization of different items (Tiede et al., 2017). The dominant bacterial phyla identified in the gut of the two amphibians were similar to other studies (Fig. 5C) (Chang et al., 2016; Huang et al., 2018), which might be explained by the stable gut environment across species (compared for instance to the external environment). The relative abundance of taxa, however, varied between the two species probably associated with gut physiology, host diet and habitat conditions (Ley et al., 2008; Tiede et al., 2017; Zhang et al., 2010). Members of the phyla Firmicutes (mainly belonging to the class Clostridia) are linked to fermentation of carbohydrates and found to be common in terrestrial animals, thus its high occurrence in *P. mascareniensis* was expected. The Asian toad has a longer gut, and the lower oxygen availability associated with this environment (in addition to the host's generalist diet), might explain the dominance of Bacteroidetes (Döring et al., 2017; Nelson et al., 2013). Bacteroidetes can also assist in metabolizing different energy sources (Flint et al., 2012). The higher proportion of members of the family Desulfovibrionaceae in the toad should be further studied since the group includes taxa that can be opportunist pathogens and produce endotoxins (Zhang et al., 2010). The absence of significant differences could be the result of the low sample size (only 3 pooled samples per species) and thus more samples should be included in future assessments. However, it should be noted that each pool included a mix of gut samples from 4 individuals and thus, the bacteria community characterized here correspond to a total of 12 individuals from each species. Although the lack of significance in alpha and beta diversity, it is worth noting that differences in abundance levels, number of unique OTUs and functional inference were found. Of noted, is the phylum Fusobacteria and the genus *Cetobacterium*, that have been found to be particularly associated to marine species (e.g., fishes) as well as *R. marina* (Zhou et al., 2020).

CONCLUSIONS

The Asian toad expansion is ongoing and comparing the recently introduced populations of *D. melanostictus* across its invaded range into different habitat types could help understand how microbiome change through the process of invasion in a contemporary scenario. Our study sites, although very anthropized and homogeneous, indicate that the toad microbiome is richer and more diverse than the one of the native species. Both species are terrestrial thus this high diversity should be associated with the toad intrinsic physiological and ecological traits but including other native amphibian species is needed to further understand these differences. Additional data from its natural range in Asia and from other invasive populations could help to better characterize the degree of variation between native and invasive populations. For Madagascar, we encourage the development of new studies aimed at characterizing skin secretions and the antifungal properties of the Asian toad's skin microbiome. Similarly, we think that it will be beneficial to further investigate the connection between the toad's diet and its gut microbiome (composition and functional roles), especially in this invasion scenario where the host may have to adapt fast as it will expand to different areas in Madagascar (colonization of different habitats).

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CHAPTER IV

Marked changes in skin bacterial communities match limited alterations in gut communities in metamorphosing anurans and urodele species from a small temporary pond

In preparation

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ABSTRACT

The metamorphosis process has several physiological, morphological and ecological alterations in amphibians but its effect depends of host species. At the same time, the symbiotic bacterial communities found in the skin and gut of individuals undergoing metamorphosis may change. Here, we compare the alterations in the skin and gut microbiota in three amphibian's species from different families (Bufonidae, Alytidae, Salamandridae) and two orders (Anuran, Urodele) that occur in the same pond and are metamorphosing at the same time in order to find the common patterns and specific differences. Overall, host identity and phylogeny had a significant effect on skin bacterial community and at lower extent in the gut. Life stage also influence these communities, especially in terms of bacterial composition and relative abundance. These changes were more marked between the aquatic and terrestrial stages that are expected to be linked with the shift in the ecology of the host and morphological and physiological alterations. The urodele seems to have bacterial communities closer to the water environment while the two anurans exhibit more similarities among them.

Keywords Bufonidae, Alytidae, Salamandridae, skin, gut, microbiome, 16S rRNA

INTRODUCTION

Amphibians are an ancient group of vertebrates constituted by three major radiations, the anurans (frogs and toads), the caudata or urodeles (salamanders and newts), and the gymnophiones (or caecilians) (Wake & Koo, 2018). Metamorphosis is present in the three amphibian orders, and although there are several exceptions (e.g. some amphibian species lay eggs that develop directly into adults' miniatures or give birth to those miniatures, a process called internal metamorphosis and direct development, respectively (Lynn, 1961), most species are linked to water for their larval phase, whereas adult life is generally conducted outside of water with some exceptions such as reproduction seasons (Duellman & Trueb, 1986; McDiarmid & Altig, 1999). The metamorphosis process is a demanding phase during the life cycle of amphibians, where an individual experiences major morphological, physiological and behavioral transformations including the gain or loss of body structures useful during the larval stage (e.g. the loss of external gills, eyelids and retinal pigments and tail, and the appearance of limbs) (Brown & Cai, 2007; Vitt & Caldwell, 2009). While larvae of urodeles are overall morphologically similar to adults (also sharing with the adults

a carnivorous diet), larvae of anurans (hereafter referred to as tadpoles) undergo more radical metamorphic changes (Duellman & Trueb, 1986; Wells, 2007).

Among the body structures that suffer the most drastic changes in both anuran and urodeles, is the skin. The skin is in direct contact with the surrounding space and in amphibians it functions as respiratory, osmoregulatory and thermoregulatory organ, as well as, constitutes the first protective shield of the host against environmental offenses (e.g., climate, habitat alterations, predators, pathogens and parasites), among other vital functions (Duellman & Trueb, 1986). In anurans, skin structure transformation starts from a simplified thin epidermis characterized by a tree-cells layer with unicellular glands that produce mucus, present in tadpoles, and turns into a complex and keratinized structure in adults composed by an epidermis, a dermis and the presence of tubercles (or warts) and a great variety of multicellular glands that can produce skin toxins and a multitude of other secretions (Brown & Cai, 2007; McDiarmid & Altig, 1999). In urodeles, the skin structure suffers similar transformations, going from a more simplified version to a stratified structure, the skin of larvae is known to possess also another type of cells, the Leydig cells, that degenerate during metamorphosis and release secretions that are suggested to contribute to the skin impermeabilization in adults (Norris & Carr, 2013; Perrotta et al., 2012).

Different amphibian classes and families exhibit different skin characteristics and produce distinct skin compounds. Amphibians host one of the most abundant and diverse inventory of skin antimicrobial peptides (AMPs), alkaloids, and other skin compounds that can function as defense against predators or as protection against pathogens (Conlon, 2011; Macfoy et al., 2005; Varga et al., 2019). In the Bufonidae family (class Anuran), the adults have a more rugous skin with tubercles and parotoid glands that usually develop during metamorphosis and can produce several noxious substances, such as tetradoxins and bufodienolides, as well as sequester skin alkaloids from its preys (Macfoy et al., 2005; Regueira et al., 2016). Some tadpoles are also able to produce both skin mucous and noxious secretions, the latter generally used to avoid predation. Within the order urodeles, some newt species were also found to harbor skin tetradoxins and alkaloids, while the salamanders are known for the secretion of alkaloids (Vences et al., 2014; Yotsu-Yamashita et al., 2007).

The digestive system of amphibians is another structure that suffers important structural and physiological alterations during metamorphosis. These modifications tends to be more evident in anurans, where the entire digestive tract of tadpoles undergoes a complete shift in morphology, histology and function, which include a massive reduction in the gut length, a change in the epithelium and the full development of the stomach and gastric glands (Brown & Cai, 2007;

Hourdry et al., 1996; Vitt & Caldwell, 2009). These ontogenetic physical changes are generally accompanied by alterations in ecological and behavior features and goes with a significant dietary shift. In general, early aquatic larval stages are active feeders until the onset of the metamorphosis process, then individuals tend to pause the feeding behavior while metamorphosing and start to feed again when metamorphosis ends (McDiarmid & Altig, 1999). Anuran tadpoles start as detritivores, microphagous or herbivorous, and become carnivorous as neometamorphs and adults (Hourdry et al., 1996). Different from anurans, urodeles suffer slightly less modifications of the digestive tube structure during metamorphosis (Hourdry et al., 1996); this is in partly due to the similar dietary preferences between urodele life stages, where individuals are carnivorous across the whole life cycle. Aquatic larvae may consume aquatic invertebrates, tadpoles or even conspecifics, and after metamorphosis, the terrestrial stages feed mostly on terrestrial invertebrates (Schriever & Williams, 2013; Wells, 2007). These differences put early aquatic stages of anurans and urodeles in different trophic levels while terrestrial stages share a more similar diet.

Symbiotic microbial communities are ubiquitous in the animal world. In amphibians, skin properties influence the microbial community composition while the latter can interplay with the cutaneous defense mechanisms of the host (Becker & Harris, 2010). Microbial communities vary with several factors including host species and life stage (Kueneman et al., 2014; Sabino-Pinto et al., 2017). Tadpoles usually have a smaller set of defenses (e.g., defense skin peptides) and during metamorphosis, amphibians enter into a highly vulnerable phase due to, among others factors, skin restructuration where individuals don't usually have their skin chemical defense completely functional yet, while fully developed adults do (Flechas et al., 2018; Langhammer et al., 2014; Woodhams et al., 2016). These functional chemical defenses include skin glands, skin peptides, among others, that can possess antibiotic and antifungal properties (Rodríguez et al., 2017). Consequently, some symbiotic bacteria can be crucial resources in maintaining host defenses during metamorphosis and across whole amphibian development. The gut bacterial communities play an important role aiding the digestion of the host and can be species-specific (Bletz et al., 2016). These microbes are fundamental for nutrient acquisition, digestion, detoxification mechanisms and immune response, improving host capacity to maintain an equilibrate and healthy metabolism (Iacob & Iacob, 2019). Meanwhile, the environment of the digestive tract, consist in a specialized tube, characterized by a narrow pH range and the anaerobic conditions that place the microbial communities in a very selective environment (Beasley et al., 2015; Ley et al., 2008). During anuran metamorphosis (and in association with

gut restructuration and dietary changes), the gut bacteria has been observed to change accordingly (Kohl et al., 2013).

A few studies have addressed how skin and gut bacterial communities vary during metamorphosis, but have mostly been focused on a single species or different populations of the same species (Ellison et al., 2018; Kohl et al., 2013; Kueneman et al., 2014; Sanchez et al., 2017). Environmental conditions, sampling site and time of sampling can have a big impact on the microbiome (Estrada et al., 2019; Kohl & Yahn, 2016; Krynak et al., 2015), therefore these variables need to be limited as much as possible. Here, we characterize the microbiome of three syntopic amphibian species, metamorphosing at the same time, across four developmental stages. We selected two anurans and one urodele belonging to families Bufonidae, Alytidae and Salamandridae, respectively. To further decrease the influence of environmental variability, we collected all individuals from a single ephemeral pond during a three-week time period. We focus on how bacterial communities vary among (1) host species; (2) developmental stages across metamorphosis for each species; and (3) compare the patterns of skin and gut. We test the following hypothesizes: i) skin and gut bacterial communities should differ among species and relative to their phylogenetic distance; ii) the urodele should present a particularly different gut community due to diet differences; iii) different developmental stages should host distinct bacterial communities, with the neometamorphs exhibiting more pronounced differences due to their distinct ecological niche; iv) variation across developmental stage should be stronger on anurans due to the complete restructuration of the gut and dietary changes.

METHODS

Study system and sampling

Two anurans (the natterjack toad, *Epidalea calamita*; and the Iberian painted frog, *Discoglossus galganoi*) and one urodele species (the palmate newt, *Lissotriton helveticus*) were sampled. We chose these species because they occur syntoptically and represent three different amphibian families and two amphibian orders. All specimens were collected from a single temporary pond in Mindelo Ornithological Reserve (41°19'17.1"N 8°44'09.6"W, Mindelo, Portugal). The temporary pond was shallow (up to 20cm of depth), had an area of circa 10 m^2 (at the beginning of the sampling), and it was without vegetation and had scarce Phyto- and zooplankton, the latter mostly composed of *Chironomus* larvae and *Cladocera*. Sampling took place between April 24th and May 15th of 2018 (across a three-week period) during the amphibian reproduction season in boreal temperate climate.

The four sampled developmental stages are described in Table 4.1. We collected ten individuals for each developmental stage and species. Neometamorphosed individuals (stage 4) were sampled already outside the water but close to the pond, and within about one week after completing the metamorphosis. All individuals were collected and brought to the laboratory. Each individual was handled with a new pair of sterile nitrile gloves and was rinsed with sterile water to remove transient bacteria. Skin swabs were collected following the protocol used in works of McKenzie and Kueneman (Kueneman et al., 2014; Mckenzie et al., 2011b). Very briefly, larvae, tadpoles and metamorphs were swabbed uniformly throughout the entire body (except for the cloacal area) during 30 seconds and the neometamorphs were swabbed on the entire ventral side (10x up and down) and 5x on each limb and foot. Water samples were collected by merging the swabs during 30s on water and moving it forward and back without touching the sediment. Individuals were euthanized in a MS-222 (SIGMA) solution, and the gut was collected. We collected a water swab each time that we collected the skin swabs and gut samples. All samples were dry-stored in sterile vials and kept at -20ºC until further processing. Samples were collected under the research permits 14692/2018/DRNCN/DGEFF obtained by the Portuguese Instituto da Conservação da Natureza e das Florestas (ICNF).

DNA extraction, amplification and sequencing

Extraction of bacterial DNA from skin swabs and whole intestine was performed using a modified protocol of Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) with a pre-treatment consisting in a lysozyme incubation step at 37°C (1h for swabs and 1h30 for guts and vortexing every 20 minutes) to break up the cell walls of Gram-positive bacteria. The V4 region of the 16S rRNA gene (hereafter 16S) was amplified and sequenced following the dual-index approach of Kozich *et al*. (2013). PCR reactions were performed in duplicate with a total volume of 12.5 µl including 0.2 µl of Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, Ma, USA), 0.25 µl of forward primer (515F: 5'-GTGCCAGCMGCCGCGGTAA-3', 10 µM), 0.25 µl of reverse primer (806R: 5'-GGACTACHVGGGTWTCTAAT-3', 10 µM), 0.25 µl of dNTPs, 2.5 µl of buffer, 8.1 µl of H_2O and 1 µl of template DNA. The amplification protocol consisted of an initial denaturation step at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10s, annealing at 55°C for 30s and elongation at 72°C for 30s, followed by a final extension at 72°C for 5 min. PCR products of the two replicates were pooled in a total volume of 25 µl, visualized on a 1% agarose gels, and purified with QIAQuick Gel Extraction Kit (Qiagen, Hilden, Germany). Successfully amplified products were sequenced on an Illumina MiSeq platform using a paired-end 2 x 250 v2 chemistry in the Max Planck Institute for Evolutionary Biology, Plön, Germany.

Table 4. 1. Selected developmental stages in the two anuran and the urodele species and an overview of the main characteristics

		Anuran			Caudata		
	Life stage	**Description (Gosner stage)**	**Main Characteristics**	**Example**	**Description**	**Main Characteristics**	**Example**
Stage 1	**Pre-metamorphosis**	**Tadpoles (G26)**	Detritivores, microphagous and herbivorous (e.g., detritus and algae, among others including coprophagy) No limbs Aquatic		**Larvae**	Carnivorous (feeds on planktonic animals and tadpoles); Cannibalism not rare Only front legs Aquatic	
Stage 2	**Early metamorphosis**	**Early Metamorph (~G38)/ larvae at the onset of metamorphosis**	Detritivores, microphagous and herbivorous (e.g., detritus and algae, among others including coprophagy) Two back legs Aquatic		**Early Metamorph**	Carnivorous (feeds on planktonic animals and tadpoles); Cannibalism not rare Four legs Aquatic	
Stage 3	**Late metamorphosis**	**Metamorph (G42)**	Four legs Tail regression Onset of the fasting period (Jenssen et al.,1967).		**Late Metamorph**	Carnivorous (feeds on planktonic animals and tadpoles); Cannibalism not rare.	

			No feeding Aquatic			Reduced gills Aquatic
Stage 4	**Post-metamorphosis**	**Neometamorph (G45-G46)**	No tail Carnivorous (mainly on insects) Terrestrial		**Neometamorph**	Carnivorous (mainly on terrestrial insects) Gills reabsorbed Terrestrial 

Samples processing and microbiome statistical analysis

Quality filtering, clustering and rarefaction was performed in QIIME 2 (Bolyen et al. 2019, https://qiime2.org). Sub-operational taxonomic units (sOTUs) were identified using the deblur pipeline (Amir et al., 2017) and taxonomy was assigned using the Greengenes 13.8 reference database (May 2013 release; http://greengenes.lbl.gov/). Chloroplast and mitochondria were removed from the dataset. Samples with less than 8,000 reads were removed and the final dataset included 175 samples (Table 2). A bacterial phylogenetic tree was generated using fasttree2 (Price et al., 2010) and alpha diversity indices were calculated in QIIME 2 (number of sOTUs and Faith's phylogenetic diversity (PD)). Samples were divided into two datasets corresponding to two distinct bacterial communities: (1) skin swab samples (n = 92) and (2) gut samples (n = 78). Water samples collected in each of the distinct sampling events were used in both datasets as controls and representative of environmental bacterial pool (n = 5).

All the statistical analyses were run in R, version 3.6.1 (R Core Team, 2019). To account for the non-normality of the data, alpha-diversity indices were compared using a Kruskal-Wallis test (K-W) followed by a multiple pairwise comparison Dunn's test with false discovery rates (FDR) correction using Benjamini-Hochberg adjustment (Benjamini & Hochberg, 1995) to identify significant differences among the three host species and water and between stages within each species. Beta diversity was calculated using unweighted UniFrac metrics based on the generated phylogenetic tree and tested using permutation multivariate analysis of variance (PERMANOVA) with 9,999 permutations to evaluate the role of each categorical factor (host species and life stages) in the two bacterial communities. If main effects were significant, post-hoc pairwise comparisons were performed between groups within each factor using the R package RVAideMemoire (Herv, 2016) with p-values adjusted with the false discovered rate (FDR) method. The community composition in the different groups was visualized with a non-metric multidimensional scaling plot (NMDS). A barplot was used to show the 10 most prevalent taxa at the genus levels and respective relative abundances across groups. All the OTUs that were not identified to the genus level were then grouped into the category "unidentified" and added to the barplot. The total number of unique and shared OTUs among species and development stages were also calculated. Core OTUs were defined as the OTUs that were present in 100% (core100; skin) and in 80% (core80; gut) of the individuals from all life stages of each species, to ensure that the OTUs found were associated with each host, and to characterize their abundance throughout development.

RESULTS

Host species harbours distinct bacterial communities

- **Skin bacterial community**

Significant differences were found among host species and water for skin sOTUs' richness and phylogenetic diversity (sOTUs: K-W, chi-squared=36.881, p<0.01; PD: K-W, chi-squared=42.299, p<0.01, Figs. 4.1A, S1A, Table S2). The anuran species (*Epidalea calamita* and *Discoglossus galganoi*) exhibited closer values (sOTUs: p=0.048, PD: p=0.024) with *E. calamita* showing a trend to lower alpha diversity and both had significantly lower values (in terms of sOTUs richness and phylogenetic diversity) than *Lissotriton helveticus* and the water (p<0.001) (Figs. 4.1A, S1A, Table S2).

In terms of community composition, host identity explained 14% (Main effect PERMANOVA, $F_{(3,96)}$=6.33, p<0.001) of skin bacterial variation (Figs. 4.2A, S2A) and pairwise comparisons showed variations among the three species (Supplementary Material Table S3). Different from *D. galganoi* and *L. helveticus*, the skin communities of the three aquatic stages of *E. calamita* were dominated by bacteria of the genus *Limnohabitans* (Fig. 4.3A). On contrary, the high abundance of *Sanguibacter* in neometamorphosed was consistent in the three species. The neometamorph stages of the two anurans were more similar in terms of relative abundance of the most prevalent taxa (e.g., *Pseudomonas, Sanguibacter, Stenotrophomonas, Variovorax,* among others) than with the neometamorph of the caudata species. The latter exhibited high prevalence of *Shewanella* and *Chlamydia*, two taxa not listed among the most abundant in the two anuran species (Fig. 4.3A). Water community exhibited species of the *Bacteroides, Pseudomonas* and *Sanguibacter* as the most abundant taxa and was overall more similar to the skin community of *L. helveticus*.

The total and unique number of skin' OTUs varied across species: *E. calamita* exhibited the lowest number of sOTUs (total=2,206; unique=283) whereas *L. helveticus* exhibited the higher values (total=4,355; unique=1,254) (Fig. 4.4A, B). The three species shared only 537 bacterial sOTUs (Fig. 4.4B).

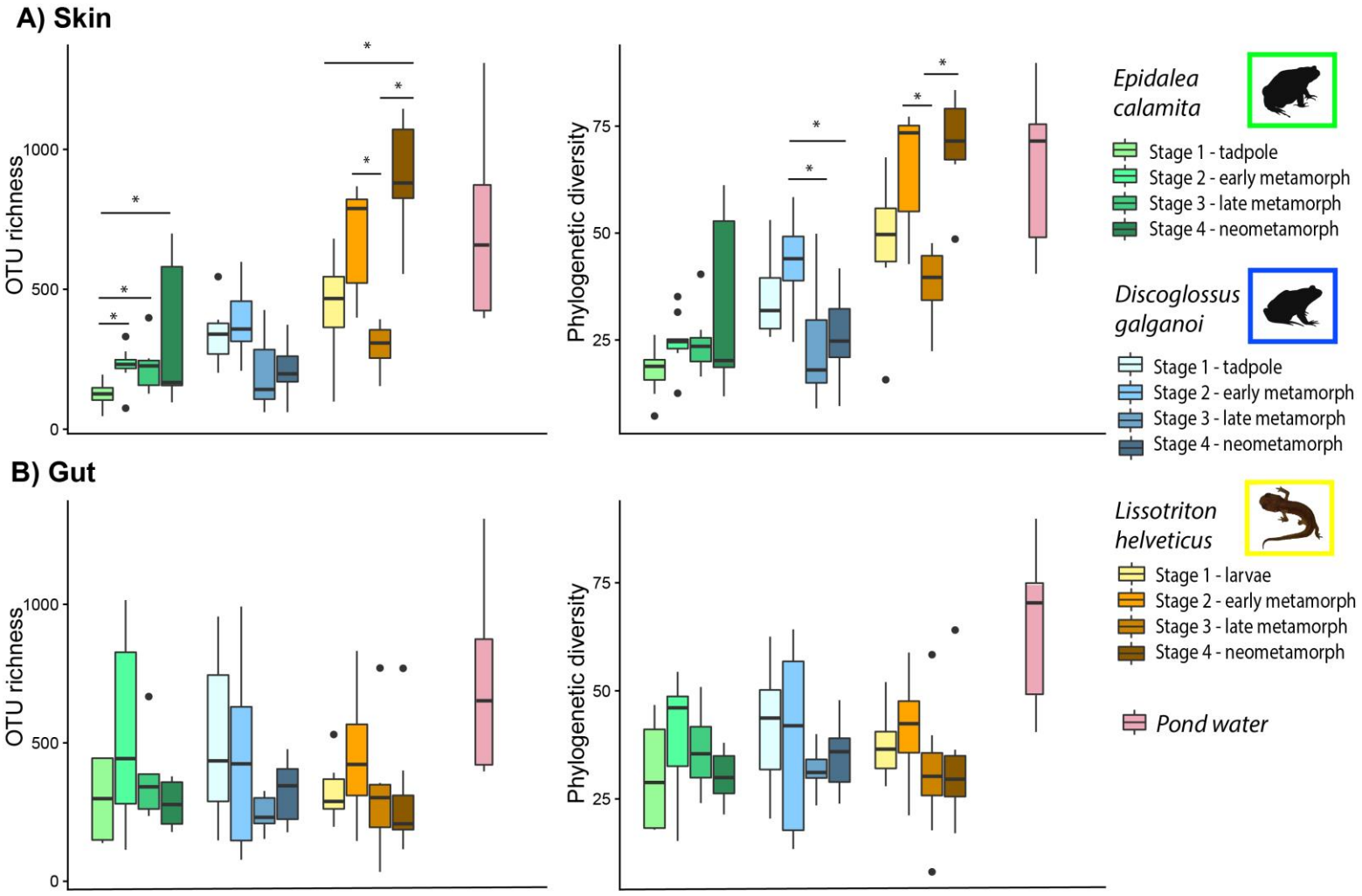

Figure 4. 1. Alpha diversity (OTU richness and Phylogenetic diversity) of (A) Skin and (B) Gut communities of *Epidalea calamita* (different shades of green), *Discoglossus galganoi* (different shades of blue) and *Lissotriton helveticus* (different shades of yellow) across metamorphosis. Pink boxplots refer to water bacterial community. Significant differences between life stages (within species) are marked with an asterisk.

The skin core100 community of stages 1-3 of the anuran species were very similar and characterized by low abundances of main taxa. Overall, the skin core100 of the anurans were similar (when the same stage was compared) in terms of taxa identity and relative abundance and both core100 communities were equally different from the skin core100 community of the urodele species (Fig. 4.6). *E. calamita* had the richer core100 community with a total of nine sOTUs. From these, six also occurred in *D. galganoi* and four in *L. helveticus*. The urodele species had two unique skin core100 bacteria that were observed only in the water. Only four of the core100 sOTUs (*Sanguibacter* sp.*, Stenotrophomonas* sp.*, Microbacteriaceae, Pseudomonas veronii*) were common to the three species and to the water community.

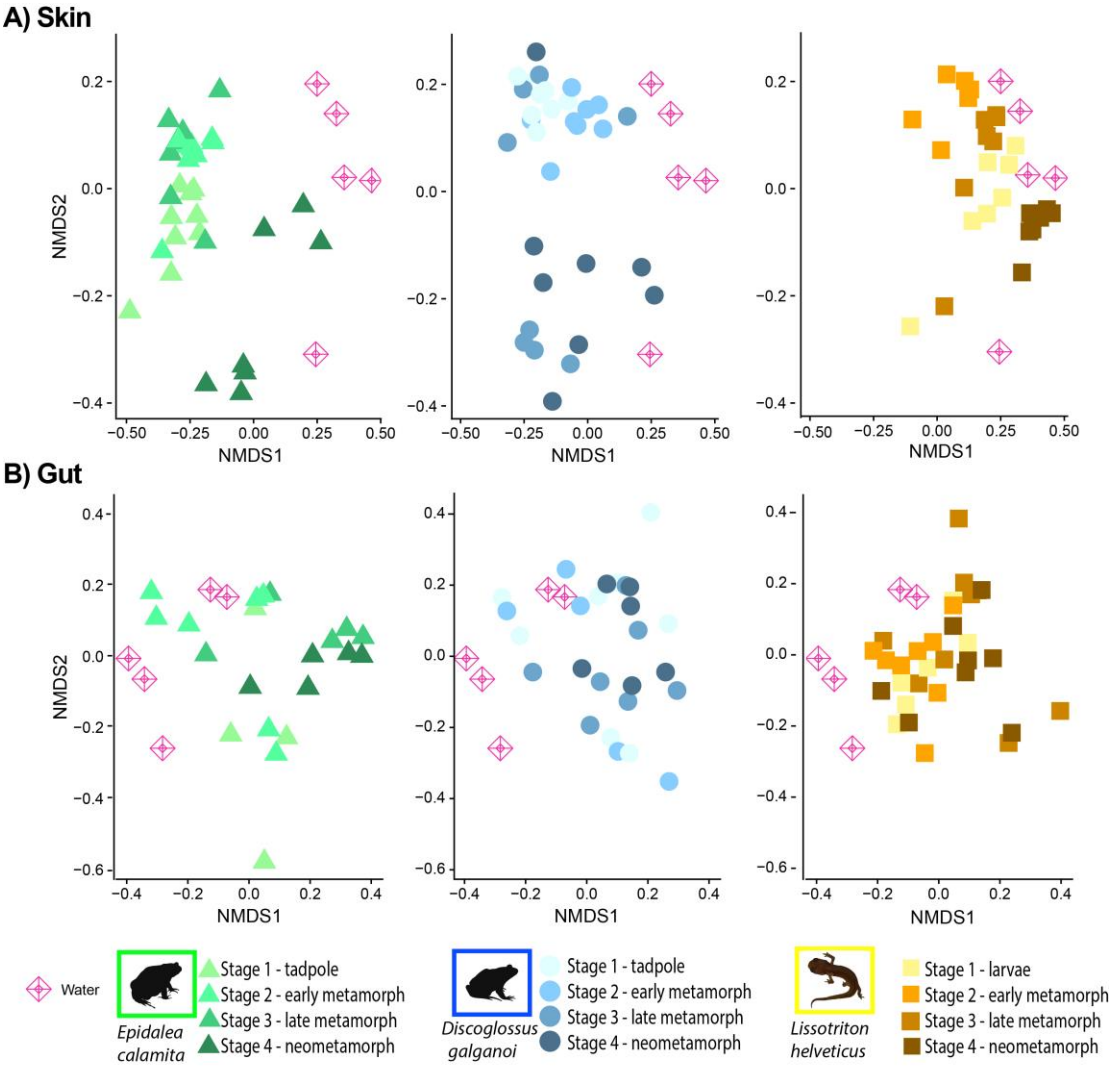

Figure 4. 2. Beta diversity of (A) skin and (B) gut communities of *Discoglossus galganoi* (different shades of blue), *Epidalea calamita* (different shades of green) and *Lissotriton helveticus* (different shades of yellow) across metamorphosis. Pink symbols refer to pond water bacterial community.

- **Gut bacterial community**

The gut communities did not present significant differences in sOTUs richness among the three species or with the water (K-W, chi-squared=6.646, p=0.08, Figs. 4.1B, S1B, Table S2). Phylogenetic diversity of gut-associated bacteria of all species was significantly lower than in water but did not differ between hosts (K-W, chi-squared=9.3519, p<0.025, Figs. 4.1B, S1B, Table S2).

Host identity explained only 7% (Main effect PERMANOVA, $F_{(3,82)}$=2.17, p<0.001) of differences found in community composition among amphibians and water (pair-wise PERMANOVA, all p<0.05, Figs. 4.2B, S2B, Tables S4, S5). Amphibians gut bacterial composition also differed from water communities (pair-wise PERMANOVA, all p<0.02, Fig. 4.2B, Tables S4, S5). Permutation test for homogeneity of dispersions showed that there were some differences between host groups ($F_{3,89}$=16.65, p<2w30.001), however, this was caused by the low number of water samples because when those were excluded no overdispersion was observed ($F_{2,89}$= 0.445, p=0.65). Gut communities carried a high proportion of unidentified taxa at the genus level, always above 60% for all species (Fig. 3B). *Bacteroides* was the most abundant taxa occurring in the three species and four stages followed by *Enterococcus* (highly abundant in *L. helveticus*) and *Ruminococcus* (highly abundant in *E. calamita*) (more details in Fig. 4.3B).

The total and unique number of gut' sOTUs varied across species: *E. calamita* exhibited the lowest number of sOTUs (total=3,369; unique=633), whereas *L. helveticus* exhibited the higher values (total=4,716; unique=1,512) and the three species shared 902 taxa (Figure 4.5). The core80 gut community of *L. helveticus* harbored the richer community with 13 core taxa, from which 4 were present in *E. calamita*, and three (*Rhodobacter* sp1, *Rhodobacter* sp2 and *Beijerinckiaceae*) in all screened species (Fig. 4.7).

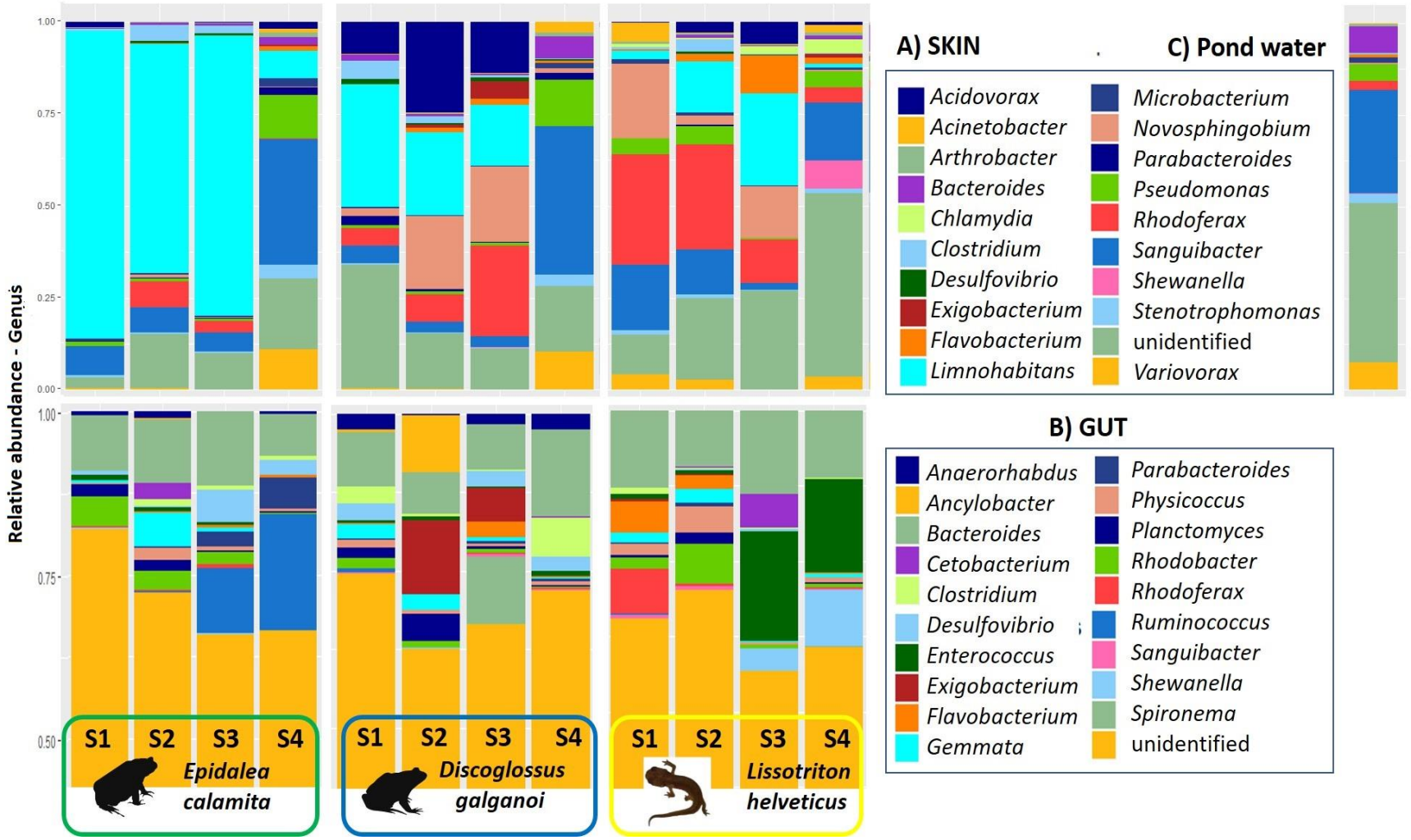

Figure 4. 3. Relative abundance of major bacterial genera from skin and gut communities pooled by species and life stages. Water taxa share the same color code as skin taxa.

Developmental stage affects skin and gut bacterial communities

- **Skin bacterial community**

Skin alpha diversity (both sOTUs richness and PD) differed among life stages (main effect, $p<0.02$, Fig. 4.1A). Differences between stages differed depending of the host although some similarities across species could indicate a trend: first an increase in sOTUs richness and PD from stage 1 to stage 2 (coincident with the onset of the metamorphosis), followed by a decrease at stage 3 (coincident with late metamorphosis and more accentuated in *D. galganoi* and *L. helveticus*) and a second increase at stage 4 that corresponds to the neometamorphs. *E. calamita* was the only host that consistently presented significant differences between the stage 1 and all the other three stages in terms of sOTUs richness but not in PD (Fig. 4.1A, Table S1). *D. galganoi* exhibited significant differences in PD between stages 2 and 3 and between stages 2 and 4 (Fig. 4.1A, Table S1); *L. helveticus* exhibited differences both in terms of sOTUs and PD between stages 2 and 3 and between stages 3 and 4 (Fig. 4.1A, Table S1).

Life stage (within each species) explained 22% (Main effect PERMANOVA, $F_{(9,96)}=3.22$, $p<0.001$) of skin bacterial variation (Fig. 4.2A). Stage 4 was the most divergent stage (Table S4): stage 4 from *D. galganoi* exhibited a significant different composition when compared with stages 1 and 2; in *E. calamita*, stage 4 was different from all the three previous stages; and in *L. helveticus*, the skin bacterial community did not vary between life stages with the exception of stage 2 and stage 4 (Table S4).

The most common taxa differed in relative abundance across stages in all species, with stage 4 being consistently the most different across species, especially in the anurans (Fig. 4.3A). The three aquatic stages of *E. calamita* were dominated by the *Limnohabitans* genus, which was reduced in the skin of the neometamorphosed (Fig. 4.3A) and a similar pattern (although less marked) was observed in *D. galganoi*. Stages 1, 2 and 3 of *L. helveticus* showed a dominance for *Novosphingobium*, *Rhodoferax*, *Sanguibacter* and *Limnohabitans* bacteria but stage 4 kept only high abundance of *Sanguibacter.* Stage 4 of the anuran species showed high abundances of *Sanguibacter* and *Pseudomonas*, which had very low abundance in the three previous aquatic anuran stages (more details in Fig. 4.3A, Sup. Mat. Figs. S3, S4, S5.

The amount of shared sOTUs in the skin between consecutive life stages showed a decrease from early to later developmental stages (Fig. 4.4C). *E. calamita* exhibited a similar number of skin unique sOTUs across the three aquatic stages but the neometamorphosed stage had almost ten times higher value (Fig. 4.4C). *D. galganoi* also exhibited lower number of unique sOTUs in

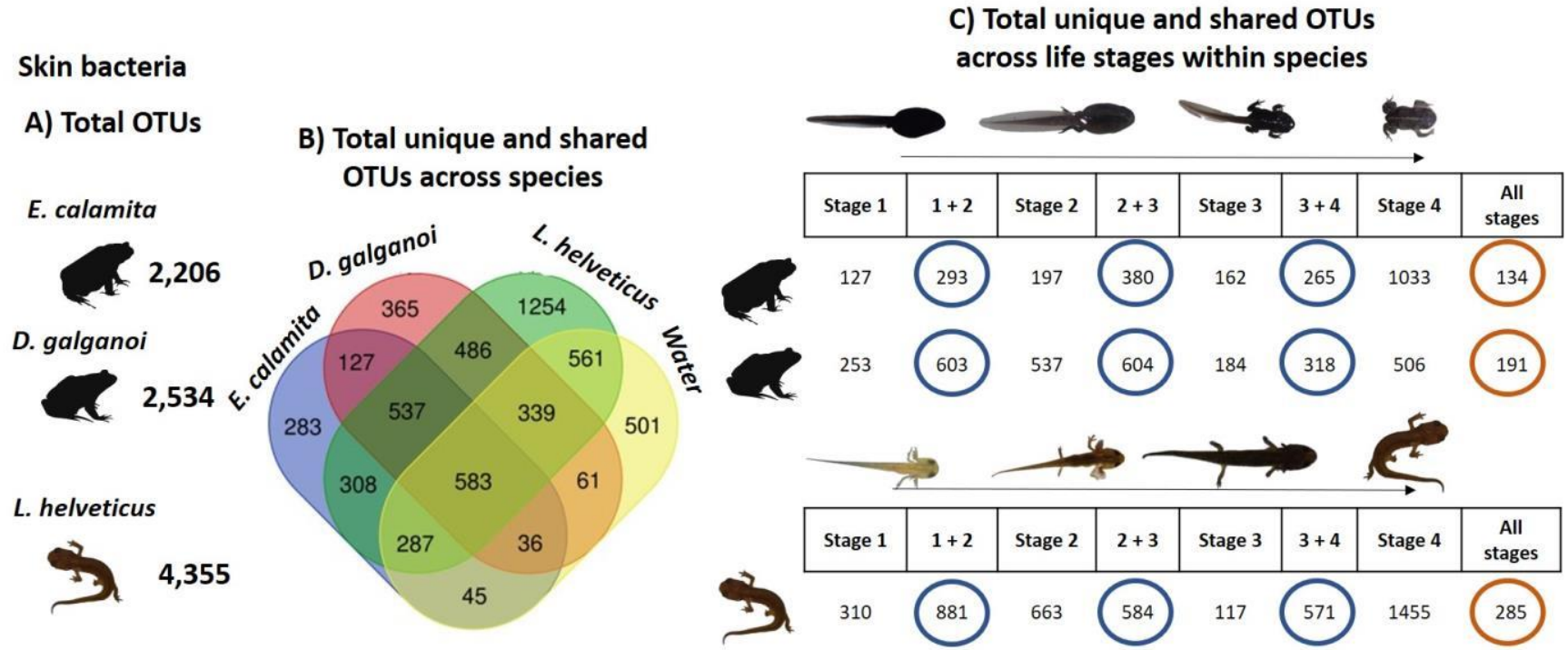

Figure 4. 4. Total number of skin' OTUs, unique and shared across species and life stages. A) Total of OTUs in each species; B) Unique and shared OTUs across species and water; C) Total of unique and shared OTUs across developmental stages within each species. Share values are represented within circles.

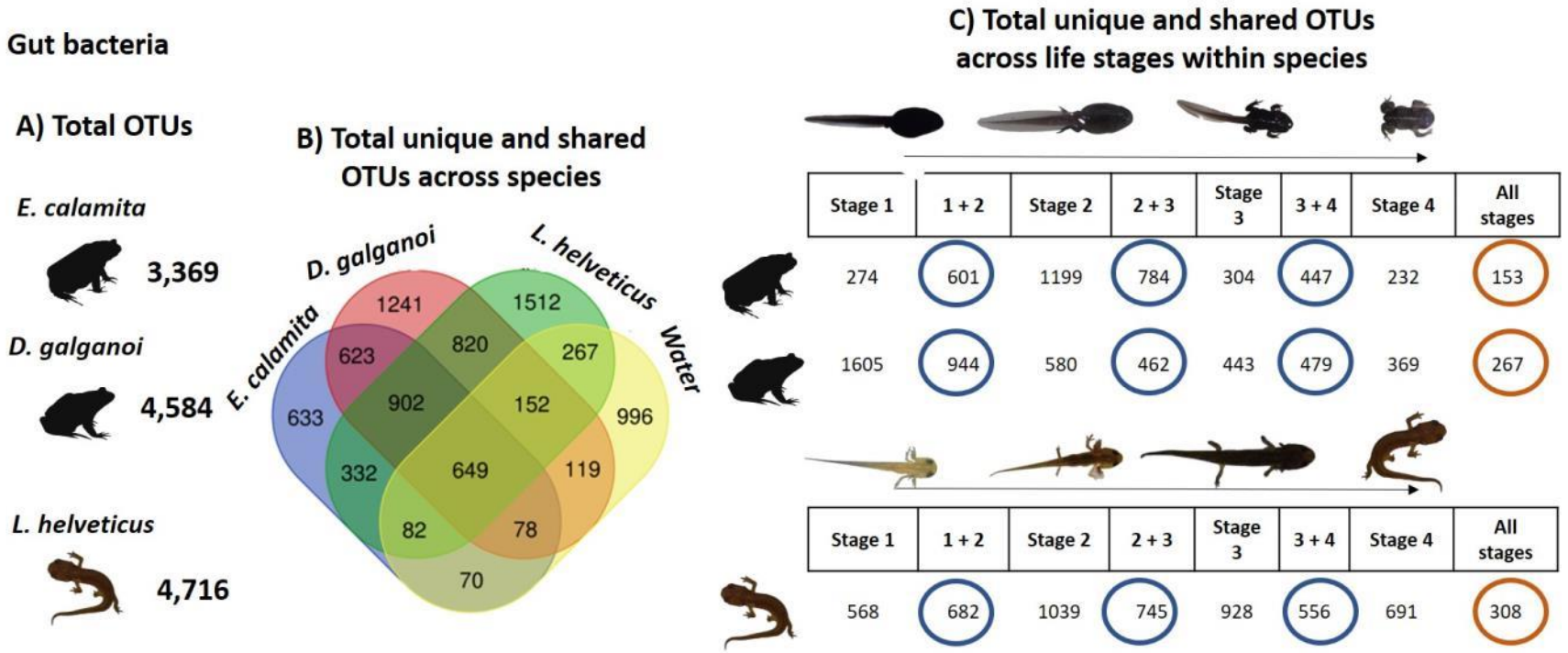

Figure 4. 5. Total number of gut' OTUs, unique and shared across species and life stages. A) Total of OTUs in each species; B) Unique and shared OTUs across species and water; C) Total of unique and shared OTUs across developmental stages within each species. Share values are represented within circles.

stages 1 and 3, with this value more than double in stages 2 and 4 (Fig. 4.4C). In *L. helveticus,* the stages 2 and 4 harbored the higher number of unique sOTUs, with stage 4 exhibiting more than the double of stage 2. Notable, all host species seem to share the same percentage of sOTUs across all the four developmental stages (Fig. 4.4C; always lower than 10% out of the total number of sOTUs) although this lower percentage is mainly derivate from the high number of unique sOTUs of stage 4.

Across development, neometamorphosed (stage 4) of anurans exhibited higher relative abundances of the core100 sOTUs, while in *L. helveticus* the core100 sOTUs were more relatively abundant in the first two aquatic stages (Fig. 4.6).

- **Gut bacterial community**

Among life stages (within each species) the gut bacterial richness did not significantly differed, although the stage 2 tended to exhibit higher values when compared with the others and this was consistently observed across the three species (Fig. 4.1B).

Life stages (within species) explained 14% (Main effect PERMANOVA, $F_{(9,82)}$=1.40, p<0.001) of variation (Fig. 4.2B). A few significant differences were observed with pairwise permanova test: in *D. galganoi*, the stage 3 was different from stages 1 and 2 (pair-wise PERMANOVA, all p<0.03); in *E. calamita*, only stage 4 was significantly different from stages 1 and 2 (pair-wise PERMANOVA, all p<0.03); in *L. helveticus*, stages 3 differed from stage 2 (pair-wise PERMANOVA, all p=0.02) (Table S5). In *E. calamita*, we observed a clustering effect of the first two stages versus the late two stages (Fig. 4.2B). This trend for the two early stages and the two late stages being more similar between them was observed in both beta diversity and taxonomic composition (Fig. 4.2B, 4.3B). Some taxa among the most prevalent ones presented different abundance levels depending of the host and stage (Fig. 4.3B): stages 3 and 4 of *E. calamita* exhibited high relative abundances of *Ruminococcus*; stage 4 of *D. galganoi* exhibited high abundance *of Clostridium*; stages 3 and 4 of *L. helveticus* exhibited high abundances of *Enterococcus* and *Shewanella*, among others (more details in Fig. 4.3B). In all species and stages, *Bacteroides* was always present with high relative abundances.

Among anurans, *E. calamita* exhibited a higher number of unique sOTUs in stage 2 (early metamorph) and the number of shared sOTUs between life stages showed a tendency to decrease across development (Fig. 4.5C). In *D. galganoi*, the first two stages exhibited the higher number of unique sOTUs and the number of shared sOTUs decreased across later developmental stages. *L. helveticus* presented the highest number of unique sOTUs at stages 2

and 3 and shared less sOTUs between stages 3 and 4 (Fig. 4.5C). Across development, stages 1 and 2 of *L. helveticus* had higher abundances of most core80 sOTUs if compared with stages 3 and 4 (Fig. 4.7). Throughout the developmental stages of the anurans, the abundance of the core80 taxa tended to decrease in stage 4 (Fig. 4.7).

Overall patterns in the skin and gut bacterial communities

Skin and gut bacterial communities exhibited different community composition depending both on species and developmental stages (Fig. 4.8; Tables S6, S7, S9). Diversity followed host phylogenetic proximity, with anurans clustering together, and the skin was more consistently different between life stages and species, whereas the gut exhibited less marked differences.

For *E. calamita*, bacterial communities differed between the two most extreme stages (i.e., stage 1 vs. stage 4) for skin ($p<0.01$) and gut ($p=0.01$). *D. galganoi* and *L. helveticus* exhibited a similar pattern, with exception that the gut communities did not differ between stages.

The bacterial community of *D. galganoi* skin at stage 1 was significantly different from the equivalent community of the other anuran, but stage 4 of the two anurans was similar ($p>0.05$). The opposite was observed in the gut communities of the two anurans species, with similar composition at stage 1 and different composition at stage 4. The skin communities of the anurans always differed from the skin community of *L. helveticus*. Stages 1 and 4 of *E. calamita* differed from the equivalent stages in the urodele species, whereas stages 1 and 4 of *D. galganoi* were similar to the one of *L. helveticus* (Fig. 4.8).

Across development and species, the two communities (skin and gut) always maintained its differences in terms of community composition and structure (Fig. 4.8).

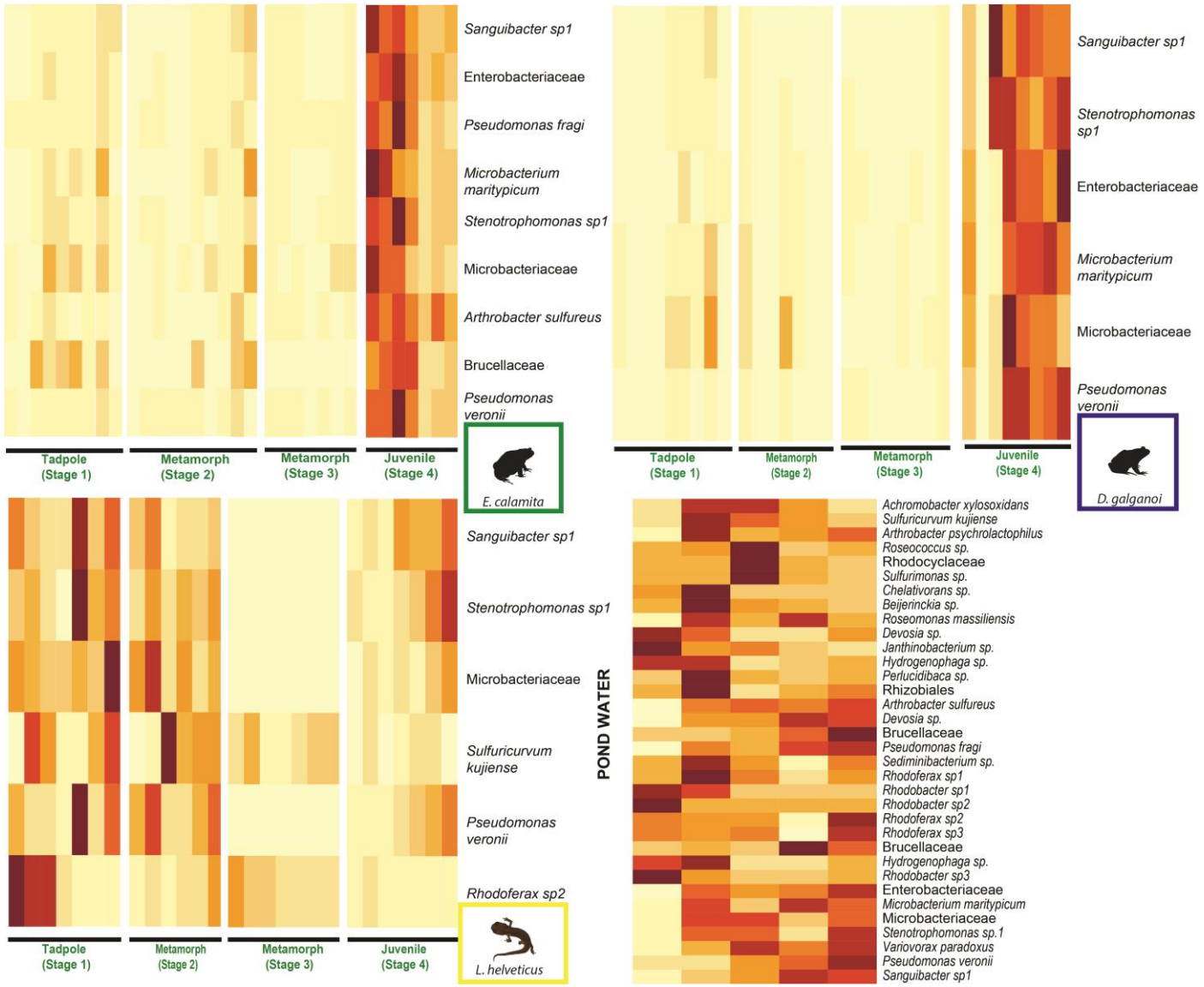

Figure 4. 6. Heatmaps of core100 skin OTUs in each analysed species and life stages. Different shades of red show abundances.

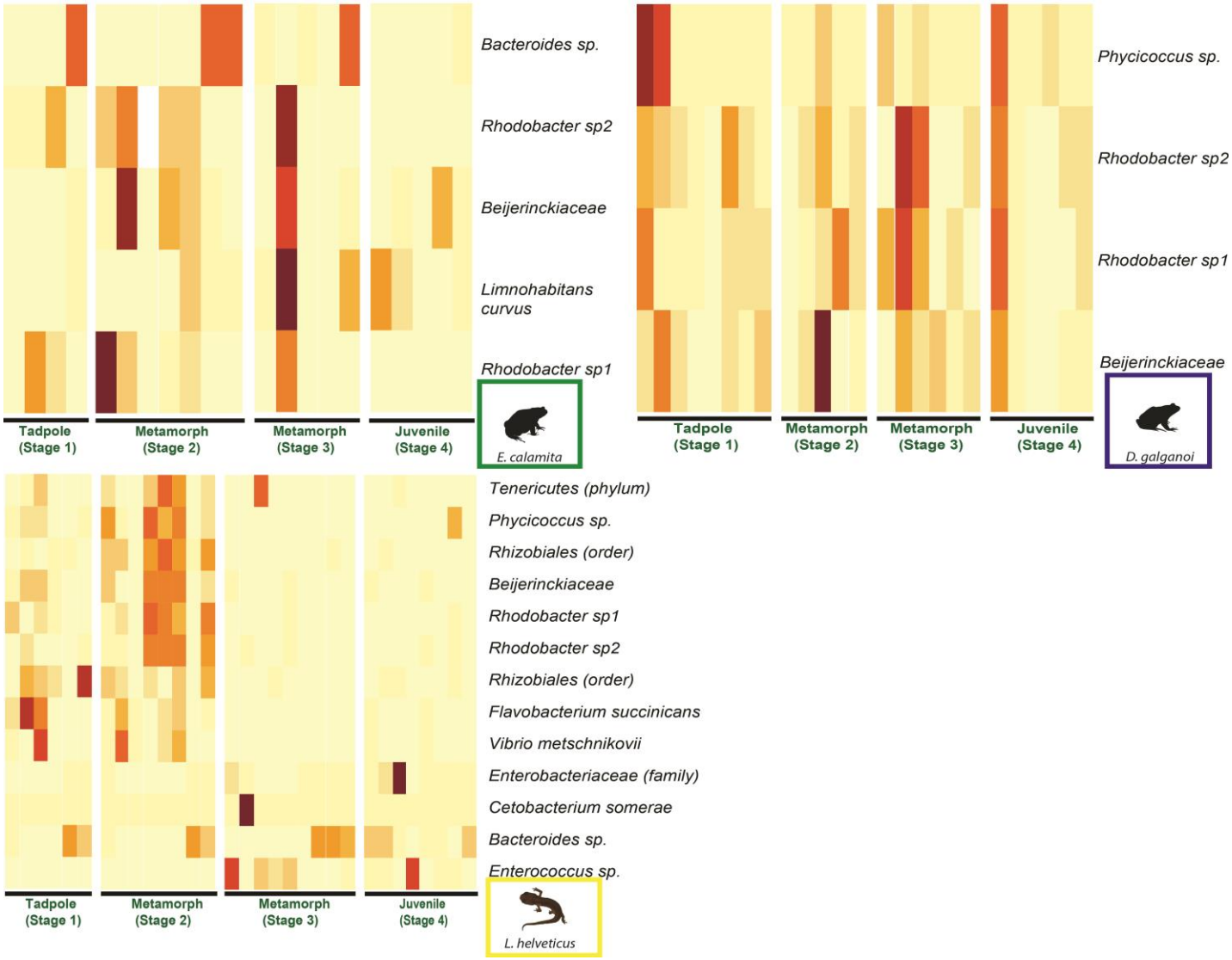

Figure 4. 7. Heatmaps of core100 gut OTUs in each analysed species and life stages. Different shades of red show abundances.

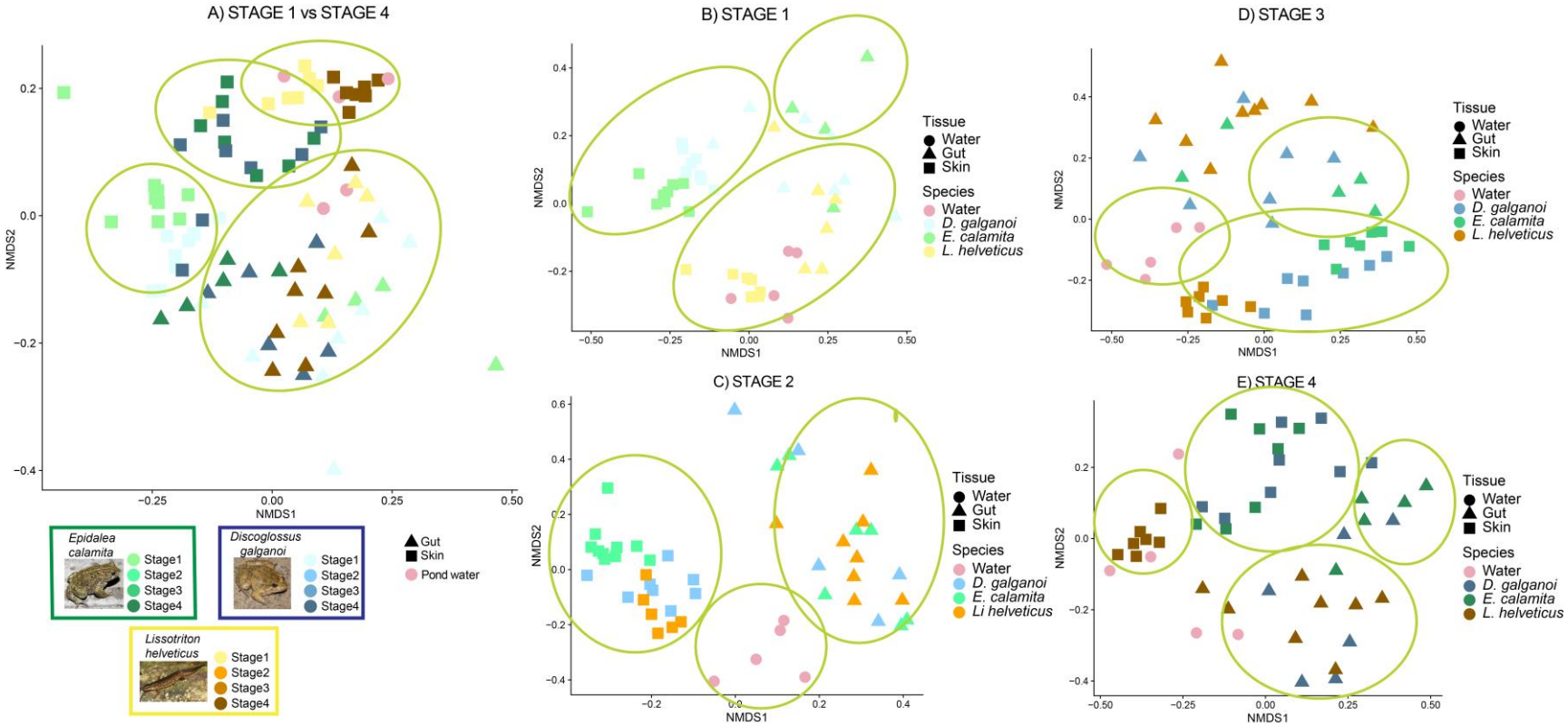

Figure 4. 8. Beta diversity comparing the skin and gut communities from the three species across stages.

DISCUSSION

Overall, bacterial communities varied with host species and life stage but patterns in the skin communities were more marked. For the skin, patterns of host-specific microbiota reported in numerous studies were confirmed to hold even with syntopic and synchronous sampling, both on average and across stages. Observed patterns indicate the urodele might uphold a less stringent filtering of environmental bacteria. The strongest differentiations along development were found at the transition to land phase (neometamorphs, stage 4), suggesting that previously reported differences from larvae to adult could be heavily linked to skin changes and not just a shift in ecology. Gut communities revealed less marked but similar trends, mostly in terms of composition. By performing synchronous and syntopic sampling across several species, this study fills several experimental design gaps left by many single-species and some multiple population studies where environmental and temporal variation was barely accounted for. The sampling of selected life stages also fills developmental gaps left by incomplete metamorphosis or adult-larvae comparison studies. This is also the first study on the gut microbiome of all study species, and among the first on Iberian amphibians' microbiomes.

Skin bacterial patterns across host species and life stages

The differences observed in the skin bacterial communities were in accordance with our first hypothesis indicating that the skin symbionts are greatly influenced by host identity either at the species or order level. We also observed that, in comparison with anurans, urodele species often holds the most dissimilar, richer and diverse communities in agreement with recent reports on the association of host-phylogeny and microbiome composition (Bletz et al., 2017a; Ellison et al., 2018; Kueneman et al., 2014). The bacterial composition of the three species also differed from the surrounding environment as it happens for most amphibian species exhibiting lower richness and diversity (Flechas et al., 2018; Kueneman et al., 2014), with *Lissotriton helveticus* skin community being more similar to the water environment than the two anurans. While differences from the environment are expected (Flechas et al., 2018; Kueneman et al., 2014), bacterial communities in urodele species have been reported to have a closer proximity to environmental microbe pools while the skin of anurans have been found to harbor some rare environmental microbes (Fitzpatrick & Allison, 2014; Walke et al., 2014a). The species shared the same spatial and temporal aquatic habitat across all the sampling period; thus, here a differential habitat effect on each species could be excluded and the observed host differences point to differences in host skin filtering of environmental bacteria between urodele and anuran. Skin properties, as for

example, the skin of *L. helveticus* that contains tetrodotoxins and some alkaloids, can affect environment suitability for bacteria (Mckenzie et al., 2011b; Yotsu-Yamashita et al., 2007).

In terms of core communities, the three species also exhibited different patterns inclusive across development. The fact that all four developmental stages of *L. helveticus* maintained always a richer skin community but a poorer core100 community than the anurans (Figs. 4.1, 4.6) may indicate that the urodele could harbor a less stringent skin environment that would enable the colonization of more bacteria. The core100 community include only OTUs present in all the individuals of each host species and across all stages so there is a possibility that these OTUs are permanent symbionts of each species (in this population). While both anurans consistently exhibited lower skin richness than *L. helveticus* (Figs. 4.1, S1)*, Epidalea calamita* carried more core OTUs (Fig. 4.5A). The lower richness and diversity could indicate a more stringent filtering than *L. helveticus*, reducing the success of colonization by other bacteria from the environment while the richer core community could indicate that *E. calamita* keeps its skin permanently colonized by high number of OTUs leaving few available niches to be colonized by environmental taxa across its development. Literature on skin properties and relation with microbiota is still limited, but a wrinkled skin and the presence of poisonous glands in both tadpoles and neometamorphosed individuals of *E. calamita* is likely to play a role. On the other side, *D. galganoi* had the poorest core100 community of the three species, with only six taxa that were also common to the other anuran species; these taxa occurred at higher abundances at stage 4 (neometamorphosed), suggesting that are permanently associated with the species, and their abundance level tends to increase across the host development.

Supporting our second hypothesis, life stage was an important determinant of skin bacterial communities as previously observed in other studies (Flechas et al., 2018; Kueneman et al., 2016) but response patterns greatly differed among species. Particularly, stage 4 was the most different, which is surely linked to an overall change in host ecology, but considering the animals were recently emerging from the water, the differences may be strongly linked to skin changes required for land life adaptation (keratinization, development of a stratified epidermis and the acquisition of secreting glands and alterations in the skin immune defenses) (Bletz et al., 2017a; Marantelli et al., 2004; Woodhams et al., 2016), leading to the consequent reassembly of the symbiotic community (Brown & Cai, 2007; Prest et al., 2018). This uniqueness was specially the result of different abundant taxa occurring at stage 4 and the high number of unique taxa found when compared with the previous three aquatic stages. We expect that due to the movement out of the pond, the individuals now in contact with soil and other terrestrial surfaces, will be exposed to new

environmental bacteria that will colonize the skin thus increasing the number of unique taxa (Loudon et al., 2014). However, when focusing only on the alpha diversity, a common trend to the two species was observed: the stage 2 (larvae starting the metamorphosis) exhibited a high number of unique sOTUs in two of the species and an increase in alpha diversity in all the species suggesting that at this stage, individuals' skin may be more suitable for bacterial colonization due to its restructuration and lower level of functional immune system (Kueneman et al., 2016). On the opposite, the stage 3 (larvae at the climax of the metamorphosis) exhibited a decrease in alpha diversity and in the total number of unique taxa in the three species suggesting that at this stage the individuals skin remodeling may present suboptimal conditions for the colonization of new bacterial taxa and the hosts also may be more susceptible to pathogens due to the lower microbial richness (Harrison et al., 2017).

The relative abundance of the most prevalent taxa was often a good indicator of life stage. For example, the taxa *Limnohabitans*, a planktonic bacteria commonly found in freshwater systems (Kasalický et al., 2013) was less abundant in neometamorphs from the three species but was found to be dominant during the aquatic stages of *E. calamita* and at a lesser extent in the other two species (Fig. 4.3A). *Discoglossus galganoi* at the three aquatic stages (pre and during metamorphosis) had higher abundances of *Acidovorax*, a common taxa occurring in amphibians skin and known to have anti-fungal properties that may contribute to the high resistance to infection although the skin of tadpoles is usually not affected by *Bd* but only the keratinize mouthparts (Woodhams *et al.*, 2015). The aquatic stages of *D. galganoi* and *L. helveticus* showed higher abundances of *Novosphingobium sp.*, a genus previously associated with mortality in frogs with fungal infections (Becker et al., 2015). On the opposite, stage 4 of the anurans exhibited high prevalence of *Pseudomonas*, an inhibitor of fungi (Becker et al., 2015; Woodhams et al., 2015), that may play an important role within the immune system since recently metamorphosed individuals are expected to have reduced immunity defenses in comparison with adults (Langhammer et al., 2014; Longo & Burrowes, 2010). More specific associated with *L. helveticus* stages, was the order Chlamydiales, consistently found at lower abundances in other urodele species where the occasional disproportional prevalence of this genus has been tentatively explained to result from microbiome disturbance or being a pathogen (Bird et al., 2018; Ellison et al., 2018; An Martel et al., 2012). Here, we observed that, *Chlamydia* spp. was found in all developmental stages of *L. helveticus,* whereas it has been found in low abundance (or absent) in the two syntopic anurans, possibly indicating that this genus could be more prone to colonize the skin of urodele species. Once again, these changes could be due to changes in the external

environment (transition from aquatic to terrestrial environment) or due to host skin changes, but the possible link with neometamorph immune functions merits more research.

Gut bacterial patterns across host species and life stages

Gut bacterial community among hosts did not exhibited significantly alterations in terms of alpha diversity and only beta diversity partly supported our hypothesis and previous observations (Warne et al., 2017) but all three communities were significantly different from water (in terms of phylogenetic diversity). Each host had different number of total, core OTUs and unique taxa indicating that at lower levels, the species-intrinsic factors (such as., gut characteristics and diet) can influence symbiotic bacterial taxa (Huang et al., 2018). The richer core community observed in the urodele compared to anurans may indicate that *L. helveticus* may have a more stable gut environment therefore allowing for a more constant and richer core community to colonize it. This high richness can also have implication in the host health since the gut core community is suggested to display important digestive and immune functions (Turnbaugh & Gordon, 2009).

The intestinal tract remodulation is known as one of the most dramatic structural changes during anuran metamorphosis where it goes from a simple and long coiled tube (in tadpoles) to a much shorter structure that includes an intestine and a stomach (in terrestrial stages) (Brown & Cai, 2007). In urodeles, these structural changes are less marked since the larva has similar feeding habits to the adults (Wells, 2007) and thus it is highly probable one of the reasons for the observed richest core community. Noteworthy, this pattern was the opposite to the skin community where the urodele exhibited a poorer core community. These results were not, however, mirrored by the richness and phylogenetic diversity, for which the three species were more homogeneous (Fig. 4.2B, Table S2),a result also recorded across developmental stages for another *Bufo* species (Chai et al., 2018). Although not significant, it is possible to observed a trend from a decrease in alpha diversity from early stages a and 2 to later stages 3 and 4. This is in line with previous studies that attributed richer gut bacterial communities to anuran larvae linked with their herbivorous diets following the same patterns found in other animal groups (Youngblut et al., 2018). The lower richness and diversity in post metamorphic stages was attributed to the diet shift to a carnivorous diet after individuals move into the terrestrial habitat and to a starvation period due to gut restructuration (Kohl et al., 2013; Vences et al., 2016). However, the same pattern occurred also in the urodele, whose larvae are carnivorous such as the adults. While here the decrease in alpha diversity may not be associated with diet shifts, it seems to be linked to the metamorphosis process where the individual may suffer a reduction in feeding activity in order to allocate energy to different physiological processes. The increase of intestine length in stage 2

(anurans) have been recorded for *Bufo* species and has been associated with an increase in gut bacterial richness (Chai et al., 2018), a results in accordance with our observations of higher alpha-diversity levels in the three amphibians. Due to the extreme environment that characterizes our study system, the pool of bacteria able to colonize the gut is expected to remain restricted which could in part have influenced the absence of significant differences among the gut bacterial communities among the three analyzed species. The pond lacked diversity of prey items, consisting mainly on algae, detritus, *Chironomus* larvae and *Cladocera*, with the last two possibly providing a richer intake to the carnivorous *L. helveticus* larvae (Huang et al., 2018).

In terms of bacterial composition and relative abundance, the two first stages of *E. calamita* were more similar between them, while the same happened for the two later stages. This may suggest that the stage 2 (early metamorphs) since it is just started the metamorphosis may still exhibit the same gut structure while start slowing down the feeding behavior while the stage 3 (tadpole at the climax of the metamorphosis) is expected to be the stage undergoing the drastic gut alterations and the fasting period (Chai et al., 2018). We observed that larvae-neometamorphs differences observed here were significantly less marked than larvae-adult differences previously reported (Kohl et al., 2013), indicating that much of the change should be due to adult diet and habitat, after the internal gut changes observed in another study (Chai et al., 2018). The clustering effect observed in the two later stages 3 and 4 in *E. calamita* supports previous findings highlighting that these two stages are more similar in terms of gut composition that may be derived from the similar gut structure in terms of length and weight (Chai et al., 2018). Moreover, stage 3 the gut is remodeling while at stage 4 it should be already completely restructured. This was also observed in *D. galganoi*, although at lower level, perhaps because some of the neometamorphs of *D. galganoi* had still a small tail bud, indicating that the metamorphosis process was still not complete and that individuals were probably not yet feeding (Chai et al., 2018). *Bacteroides* (a genus commonly found in amphibians gut) was very abundant across all species and stages (Fig. 4.3B) (Kueneman et al., 2014) but with high abundance in stages 3 and 4 of the two anurans highlighting its predominant in terrestrial stages previously observed (Chai et al., 2018) while during metamorphosis (stage 3) it can been associated with the capacity to metabolize fat and protein (Wu et al., 2011) and it can increase during fasting periods (Sommer et al., 2016). *E. calamita* individuals at stages 3 and 4 showed a high prevalence of *Ruminococcus* that are an important gut microbe and commonly found in omnivorous hosts (La Reau et al., 2016). Differently, *Enterococcus* was very abundant in stages 3 and 4 of *L. helveticus*, which is both a gut commensal but also an opportunistic pathogen (Byappanahalli et al., 2012). *L. helveticus* was the species that had more shared OTUs across the four developmental stages, suggesting a

richer and more conserved symbiotic community, which can be associated with the limited diet shifts that are expected to occur in urodeles species being in accordance with our hypothesis.

Comparison of skin and gut communities at the two most extreme stages of the three species

We observed that the skin communities' patterns across the metamorphosis process were very different from gut communities. Few studies have evaluated both communities and even fewer have compared three species from the same pond while characterizing developing stages at the time the metamorphosis process is happening. The skin communities exhibited the stronger clustering patterns during the sampled ontogenetic phases, being in accordance with previous works (Demircan et al., 2018), while the gut communities in general lacked significant differences or mainly exhibited smoother tendencies. When comparing the two communities, it is clear that in this case, the gut responses were less host species-specific than the skin community. Comparing the most extreme stages evaluated here, the early aquatic and early terrestrial stages (1 and 4 respectively) that represent the aquatic and early terrestrial phases we could highlight some patterns: i) *L. helveticus* skin communities from both stages (1 and 4) are more similar among themselves than to the same stages from the two anurans; ii) skin communities of tadpoles (stage 1) of both anuran species are highly similar among them and less similar to the same stage of the urodele; iii) skin communities of neometamorphosed anurans (stage 4) are highly similar among them and different from an earlier ontogenetic stages (stage 1); iv) the skin communities of all neometamorphosed (anurans and urodele) are more similar among them than comparing with the skin communities of the aquatic stages of each species; v) the bacterial communities from the gut are less specific, although there is a tendency for the gut of *L. helveticus* to vary less during ontogeny than the gut communities of the two anuran species across the two stages. Comparing both skin and gut communities from species co-occurring in the same pond we can conclude that species and life stage have a major role in shaping the symbiotic bacteria. Both host and life stage are a good predictor for the bacterial communities observed in the skin of the anuran species and gut communities are more stable across development especially in urodeles.

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AUTHOR CONTRIBUTIONS

BS and AC design the laboratorial experiment; BS performed the fieldwork, laboratorial experiment and sample collection; BS, FMSM and JSB analyzed the data and prepared the final data plots; BS and AC wrote the first draft of the manuscript; all authors revised and contributed to the final versions of the manuscript.

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CHAPTER V

The effect of water source on tadpole microbiome from different species in a short-term experiment using 16S metabarcoding

In preparation

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ABSTRACT

Symbiotic bacterial communities associated to amphibians are influenced by several host-intrinsic and environmental factors. To better understand the single effects of each factor, laboratorial settings are a good alternative to sampling natural populations. Here, we assess the effects of the exposition to different water sources in the skin and gut microbiome of tadpoles from two amphibian species. We observed that the response to water environment is greatly associated to host identity even at these early aquatic stages. In the species collected from the waterbody with lower bacterial diversity, the richer water source significantly increased phylogenetic diversity and composition of both communities. The other species exhibited less marked responses with both skin and gut communities showing the same alpha-diversity patterns independently of the water source, suggesting that water environment did not influence its bacterial diversity but only in terms of composition and structure.

Keywords 16S rRNA gene, amphibians, bacteria, translocation, *Pelophylax perezi*, *Bufo spinosus*

INTRODUCTION

The study of the associations between microbial communities (or microbiome) and multicellular hosts has received much attention recently due to their expected role in host immunity and physiology. In general, a poorer or imbalanced microbiome community (dysbiosis) likely increase host susceptibility to pathogens or disrupt house-keeping physiological functions (Iacob & Iacob, 2019). These communities have been studied in numerous vertebrate and invertebrate hosts (e.g. Gil-Turnes et al. 2016, Knutie et al. 2017, 2018) and, in the case of amphibians, particular focus has been given to the study of skin and gut microbiomes. The intrinsic properties of the amphibian skin (e.g. secretions, peptides) seem to affect the composition of cutaneous microbial communities (Conlon, 2011) and, as a consequence, it can enhance its susceptibility to pathogens (Bletz et al., 2013; Lam et al., 2010), whereas in gut, symbiotic bacteria have shown to play a major role in homeostasis, digestive efficiency and health maintenance (Jiménez & Sommer, 2017). Both these communities are affected by changes in biotic and abiotic environmental factors as well as host-intrinsic characteristics (Jiménez & Sommer, 2017). Studies that characterize amphibian skin-associated bacteria showed these communities can vary according to host identity (including species genotype, ecology and behavior), habitat features

and alterations (e.g., water changes, soil use and human occupation) and climate conditions, among others (Bletz et al., 2017b; De Assis et al., 2017; Krynak et al., 2015; Kueneman et al., 2019; Kueneman et al., 2014; Mckenzie et al., 2011a; Sabino-Pinto et al., 2016, 2017). Particularly, host identity has been found to be among the most important factors influencing skin bacterial composition and structure in amphibians (Kueneman et al., 2014) where the bacterial richness and abundance of key taxa strongly differs among species suggesting that each host skin may select for different bacteria (Walke et al., 2014b).

Similarly, habitat characteristics (e.g. water body), dietary preferences and gut mucosal structure can influence gut-associated communities affecting bacterial composition, structure and functions (Colombo et al., 2015; Correa et al., 2020; Vences et al., 2016). Amphibians are assiduous inhabitants of freshwater ecosystems, one of the most diverse but also threatened ecosystems due to anthropogenic pollution, land use and climate change (Ormerod et al., 2010). Therefore, it is expected that any variation in water parameters (e.g., temperature, pH level, salinity, nutrients and/or chemical pollutants) will shape the environmental bacterial pool that in turn can affect the bacteria composition associated with amphibians' skin and gut environments (Krynak et al., 2015). Amphibian species that are found in both pristine and anthropogenized habitats present symbiotic communities that may reflect their occurrence in the different environments (Huang et al., 2018; Hughey et al., 2017). Species that make contact with new habitat conditions face a shift in their microbial communities, which become more similar to the bacterial composition of the individuals from the new source, and in case of the gut communities, it was also observed a change in their predicted functions (Bletz et al., 2016). Moreover, when in contact with a lower quality environmental scenario, the pool of bacteria can be reduced and host skin microbiota may exhibit a poorer bacterial community, distinct composition or functional richness than the same host species at higher quality environments (Chang et al., 2016; Huang et al., 2018). Studies on skin and gut microbiomes of amphibians usually focus on the effect of host species, site location or habitat type on microbial community variation and mostly has been done only in adult individuals (Mckenzie et al. 2011, Kueneman et al. 2014, Chang et al. 2016). Within the habitat, water characteristics (.e.g., nutrients, pollutants, temperature, pH), while host-intrinsic factors can include age and trophic positions that are expected to affect these communities since early (aquatic) life stages in amphibians (Correa et al. 2020, Kohl & Yahn 2016, Mu et al. 2018). By being continuously exposed to water, the composition of skin and gut-associated communities of tadpoles can unveil time-specific dynamics associated to habitat characteristics and water alterations, although it is not yet fully understood how stable are these communities and how they respond to external perturbations (Rebollar & Harris, 2019). Their stability, in terms of bacteria

composition, abundance and functional signatures, and the effect of the exposure to a new environmental microbial reservoir may also provide new insights on microbial relationships (Krynak et al., 2015). Understanding which factors are important in defining the structure and dynamics of the host-bacterial communities can help clarifying ecological networks in amphibian-bacterial associations within specific environmental scenarios, including providing clues of how the host may respond to these alterations (Lam et al., 2010). Previous studies aimed to address this "habitat effect" by translocating populations or species to different water ponds in a natural setting (Bletz et al., 2016; Lokmer et al., 2016). However, in such cases, several environmental factors could not be controlled. Using a laboratory setting, where all other variables are more easily controlled it is possible to assess the changes on bacterial communities that most likely result from the exposure to a new water environment, and the time frame in which they take place.

Our work explores the changes in skin- and gut-associated bacteria in tadpoles through time, exposing them to their 'natural' water (native) or to a new water source (translocated) under the same controlled laboratory conditions. For this, we selected two widespread species occurring in Portugal, the Iberian green frog *Pelophylax perezi* (López-Seoane 1885) and the European common toad *Bufo spinosus* (Daudin 1803). Both species can be found in different habitat types, from retrodunal systems to wetlands, forests, rural and anthropogenized habitats (Speybroeck et al., 2016), inhabiting ponds, rivers and other large waterbodies. Particularly, *B. spinosus* uses preferentially low-flowing waterbodies either temporary or permanent, whereas *P. perezi* has a preference for using permanent ponds within wetlands (Richter-Boix et al., 2007). Although *P. perezi* can also forage throughout the entire water column, *B. spinosus* and *P. perezi* tadpoles are generally bottom-dwellers and present an omnivorous diet, consuming detritus, algae, plankton, aquatic plants, and arthropods (Diaz-Paniagua, 1985, 1989). The skin of the tadpoles of both species is smooth, but the skin of *B. spinosus* tadpoles is known to produce toxins to avoid predation, a characteristic that is maintained also in adulthood. The main goals of this study were to understand if and how fast the bacterial communities of tadpole skin and gut changed when maintained in two different water sources (native vs translocated), and if the two species would show similar response patterns in the symbiotic communities. Lastly, we want to compare the response patterns of both skin and gut communities since they are often not addressed together. Through time we expected that i) the host identity would influence the symbiotic communities; ii) the water environment would have a significant impact on the tadpoles' microbiome, with individuals exposed to a new environment (translocated) experiencing a shift in their microbial

community, and that iii) the skin and gut bacterial community will show different response patterns.

MATERIAL AND METHODS

Field Sampling

Sixty-six tadpoles (at approximately Gosner stage 25; Gosner 1960) of *Pelophylax perezi* and of *Bufo spinosus* were collected on the same day in May 2017. *P. perezi* tadpoles were collected in Gafanha da Boavista (Aveiro District, Portugal; 40°36'16''N, 8°41'48''W), from a freshwater stagnant waterway with an average of 60-80 cm of depth, surrounded by agricultural fields, whereas *B. spinosus* tadpoles were collected in Lousada (Porto District, Portugal; 41°16'23.8''N 8°18'26.8''W), from a freshwater lotic stream with an average depth of 30-40 cm, surrounded by vegetation and agricultural pastures. Tadpole sampling was performed under a research permit provided by the Institute for Nature Conservation and Forests – ICNF (17105/2017/DRNCN/DGEFF). At the same day, about 20L of water per waterbody where the tadpoles were collected was collected and transported to the laboratory to fill the aquaria for the experiment. Two swabs from each waterbody were also sampled to determine the baseline bacterial environmental community for day 0.

Experimental Design

- **Reciprocal Translocation**

We carried out a 2 x 2 factorial design experiment over four weeks to evaluate the effects of reciprocal translocation on skin and gut bacterial communities of the tadpoles of *B. spinosus* and *P. perezi,* separately exposed to their native or translocated water (Fig. 5.1). Within each experimental group, two aquaria (replicates) were filled with 4L of water collected at each waterbody, for a total of four aquaria per species (i.e., two with native water and two with translocated water; Figure 1). At Day 0, a total of 15 tadpoles of each species were randomly assigned to each of the four aquaria. The aquaria were maintained under controlled laboratory conditions (12h light cycle at 24ºC room temperature) and tadpoles were fed with tropical flakes TetraMin® (Tetra, Melle, Germany) *ad libitum* every two days.

The water of the aquaria was changed every week (i.e., on days 7, 14, 21) using water freshly collected at each site (Gafanha or Lousada). Specifically, the water in each aquarium was partially replaced each week by mixing 2,8L of the newly-collected water with 1,2L of water where tadpoles have been exposed in the last seven days (corresponding to a ratio of 70:30). We also included

a negative control group to monitor the potential effect of the laboratorial experimental conditions, which consisted in one aquarium filled with water from each waterbody, kept without tadpoles throughout the experiment but subjected to the same weekly partial water replacement (Figure 1). From each water source (Gafanha and Lousada), a swab sample was collected from the newly collected water at days 0, 7, 14 and 21 and from each experimental group, a water swab was collected at days 7, 14 and 21 prior to water mixing.

- **Tissue sampling**

On Day 0, after collection of tadpoles and transportation to the laboratory, we rinsed six individuals from each species with sterile water to remove transient bacteria, swabbed their skin with a sterile swab and euthanized them in a solution of tricaine methanesulfonate (MS-222, SIGMA) to collect their gut. Thereafter, on days 7, 14, 21, 28, three tadpoles per aquaria were collected, swabbed and sacrificed (following the same process described above) for a total of 24 tadpoles each week i.e., six per species per water treatment (Fig. 1). All samples were stored at -20ºC until further processing.

- **DNA Extraction, Amplification and Sequencing**

DNA was extracted from swabs and gut tissue samples using a Qiagen DNeasy Blood & Tissue Kit protocol (Qiagen, Hilden, Germany) with an initial lysozyme incubation step at 37ºC (Belden et al., 2015). Bacterial DNA was amplified targeting the V4 variable region of the 16S rRNA gene using the barcode primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (10µM) (Caporaso *et al*. 2010b). PCR amplifications were performed in duplicate with a final volume of 12.55 µl each, including 0.2 µl of Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, Ma, USA), 0.25 µl of each primer (at 10 µM), 0.25 µl of dNTPs, 2.5 µl of buffer, 8.1 µl of H_2O and 1 µl of template DNA. The amplification protocol consisted in an initial denaturation step at 98ºC for 1 min, followed by 30 cycles of denaturation at 98ºC for 10s, annealing at 55ºC for 30s and elongation at 72ºC for 30s, and a final extension at 72ºC for 5 min. Amplicons of both PCR replicates were pooled, visualized on 1% agarose gels, and purified with QIAQuick Gel Extraction Kit (Qiagen, Hilden, Germany). Samples were sequenced using paired-end 2 x 250 v2 chemistry on an Illumina MiSeq sequencing platform using a dual-index approach (Kozich et al., 2013) in Plön, Germany.

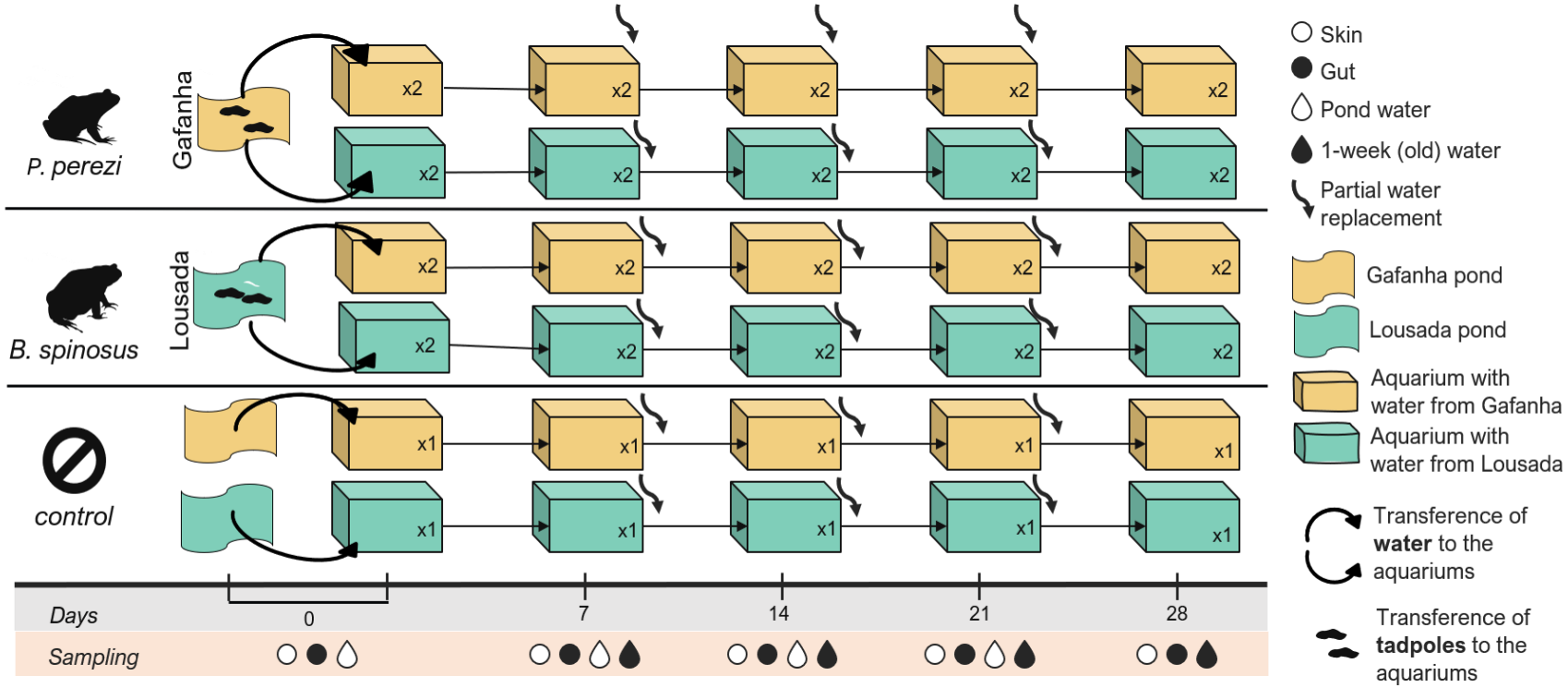

Figure 5. 1. Graphical representation of the 4-week reciprocal translocation experiment carried out in this study using *Pelophylax perezi* and *Bufo* spinosus tadpoles. The experimental design consisted in four experimental groups (2 source waters x 2 host species) and one control group for each source water (aquaria without tadpoles). The figure displays the number of replicas per experimental group (x1, x2), the sampling events (Days) and the four type of samples collected at each day – skin (open circle), gut (closed circle), newly-collected water from the waterbody (white drop), and water collected from the aquarium (black drop). Three tadpoles were sampled weekly from each group replica, where skin swabs and gut were collected. At the sampling days 7, 14 and 21, we partially replaced the water of the aquaria (wave arrow), by mixing freshly-collected water from the waterbody (70%) and the 1-week water of the respective aquarium (30%), to which tadpoles were exposed in the following seven days (see Methods for details).

Metabarcoding Data

- **Sequence Processing**

The bioinformatic tool QIIME2 (Bolyen et al., 2019) was used for sequence processing and OTU identification. Due to the typical lower quality of reverse reads (Kwon et al., 2013), only the forward reads were used for the downstream analysis; sequences were filtered following the criteria: absence of Ns within the sequence, absence of barcode errors, and exclusion of reads containing at least three consecutive low-quality nucleotides. Sequences were clustered into sub-operational taxonomic units (sOTUs, herein called OTUs) following the deblur workflow (https://github.com/biocore/deblur) (Amir et al., 2017). Sequences were trimmed to 150 bp and OTUs with less than 10 reads were excluded. The resulting OTUs were assigned to a taxonomic group using Greengenes 13.8 as reference database (May 2013 release; http://greengenes.lbl.gov/). Non-bacterial taxa (e.g.: Mitochondria and Chloroplast) were removed from the dataset. PyNAST (Caporaso et al., 2010) was used to align the OTUs sequences and FastTree (Price et al., 2010) was used to computed a phylogenetic tree. All OTUs with less than 0.001% of the total reads of all analyzed samples were excluded (Bokulich et al., 2013). A final OTU table including all the samples rarefied to 1700 reads was obtained.

Data Preparation

The microbial dataset was organized into a phyloseq object (Mcmurdie & Holmes, 2013) which included four types of information: OTU table, taxonomic annotations of each OTU, sample metadata (including experimental groups and water origin), and a phylogenetic tree (Mcmurdie & Holmes, 2013).

The final OTU table was obtained performing some additional steps: i) the sum of the read counts (pre-rarefied at 1700 reads) obtained from the two swabs collected at day 0 of each waterbody (Gafanha or Lousada) separately, and used those communities as the baseline of all groups; and ii) at each week, we simulated the water environment of the 10 aquaria by randomly picking 30% of the OTUs observed in the swab samples collected from each aquaria (corresponding to the 7-old day water) and 70% of the OTUs resulting from the newly-collected water at the respective waterbody (Gafanha and Lousada) that were used to replace 70% of the water medium each week, and summing them at the end. From this final dataset, we calculated the phylogenetic diversity of all samples. Data preparation and all statistical analysis were run in R studio (Version 3.6.1, R Core Team, 2019) using the following main packages: vegan, phyloseq, reshape,

picante, ggplot2 and lme4 (Bates et al., 2015; Kembel et al., 2010; Mcmurdie & Holmes, 2013; Oksanen et al., 2017; Villanueva & Chen, 2019; Wickham, 2007).

Statistical Analysis

Before testing our hypotheses, we first investigated if there were differences in the bacterial communities between the two water sources (Gafanha and Lousada) at the beginning of the experiment, and characterized the communities in each aquarium water (experimental groups) over time in relation to the source to understand if the tadpoles were exposed to significantly different communities in the two water sources and to confirm that the water from each experimental group were similar for the two species and representative of the source waters (Gafanha and Lousada). For this, we explored the variation in bacterial α and β diversities using phylogenetic diversity and Unweighted unifrac distances, respectively, at each waterbody (Gafanha and Lousada) from day 0 and in the water mixtures used in the experiment at days 7, 14 and 21 (Fig. 1). Day 28 was excluded because it was the last day of the experiment and thus, the tadpoles would not be exposed to that and no new water was collected at that day. Due to low sampling size of water samples, direct comparisons were performed for α-diversity using a line plot, while non-metric multidimensional scaling was used to plot the β-diversity. The relative abundance of the ten most abundant taxa (at the Phylum and Family levels) in the water samples were visualized in a barplot, organized by water source and sampling event (day). After verifying the differences between water sources, we assessed the variation in skin and gut microbiome in each species when exposed to native or translocated water over time. Generalized Linear Mixed Models (GLMMs) and Permutational Multivariate Analysis of Variance (PERMANOVA) were used to analyze respectively the α and β diversities of the tadpoles of each species and community (skin or gut) separately. In both cases, a hierarchical design was used, with “water origin” (native / translocated) and “sampling day” (0/7/14/21/28) as fixed effects. Group replicates nested within the variable “water origin” were included in GLMMs as random effects. A full GLMM was performed to test the individual effect of each factor and their interaction. The GLMMs were fitted and plotted using lme4 (Bates et al., 2015), vegan (Oksanen et al., 2017), ggplot2 (Wickham, 2016) and phyloseq (Mcmurdie & Holmes, 2013) packages in R. To understand the variations in abundance levels of the most prevalent OTUs in each experimental group, we plotted the 10 most abundant OTUs (at phylum and class levels) in for each group across the sampling days.

To identify the differences in the OTU uniqueness and abundance levels, we focused only in the initial point (day 0) and the final point (day 28). We performed a Linear Discriminant Analysis Effect Size (LEfSe) analysis with default parameters (LDA score > 2.0, α=0.05) (Segata et al.,

2011) to determine which OTUs were driving the differences between experimental groups (host and water source) at the two sampling events (day 0 and day 28). The scores of this analysis are an estimate of the effect size of features (in this case, OTUs) that are differentially abundant among the categories tested. We also calculated the total unique OTUs and the OTUs shared between each experimental group within and between species at days 0 and 28. Finally, we calculated the total unique and shared OTUs within each species across the three experimental groups (day0, day28-native, day28-translocated) in order to understand how many OTUs were constantly associated to each species that were not lost after the exposition. From those, we also identify the OTUs that were associated to all the individuals from each species (core100) in the three experimental groups.

RESULTS

Water microbiome

Differences in bacterial phylogenetic diversity and composition were observed between the water collected at the two source water bodies (Supplementary material Fig. S1), with water source collected from Gafanha showing higher α-diversity across all the sampling days. Some variability in α-diversity was observed within each collected water throughout time, but the pattern was maintained also in the water collected from the experimental groups (e.g., Control, *Bufo spinosus*, *Pelophylax perezi,* Fig. S1A-C) indicating that the water where the tadpoles were exposed was a good representative of the collected water from each site. A few exceptions were: the Gafanha water collected from the aquaria hosting *B. spinosus* tadpoles at days 7 and 14 (Fig. S1B); the Lousada water of the aquaria hosting *P. perezi* tadpoles at day 14 (Fig. S1C). In terms of β-diversity, water samples of different source waters and across sampling days could be grouped consistently by origin (Gafanha or Lousada) and day (Fig. S1D), thus indicating that the two species were exposed to similar water conditions (e.g., bacterial pool) over time. In terms of bacterial composition, the two source waters (collected at Gafanha and Lousada) showed some differences in the relative abundance levels of the most prevalent taxa (Fig. S2). At the phylum level, both carried high abundance of Proteobacteria and Actinobacteria across the sampling days, but the water collected at Gafanha also exhibited high abundances of Cyanobacteria, Firmicutes and Verrucomicrobia (Fig. S2A). At the family level, the differences between source waters were maintained, with water from Gafanha exhibiting higher variation in taxonomic diversity over time (Fig. S2B).

Host microbiome exposed to native and translocated waters

On Day 0, the skin microbial communities of *B. spinosus* tadpoles were significantly more diverse than in *P. perezi*, whereas the gut communities were similar between the two species (Fig. S3).

Over the four-week experiment, we observed significant differences in phylogenetic diversity between the host species for both skin (Fig. 5.2A; Table S1A-1B) and gut (Fig. 5.3A; Table S1C-1D) microbial communities when exposed to different water sources across days. *B. spinosus* tadpoles exposed to translocated water (Gafanha) exhibited a significant increase on skin bacterial phylogenetic diversity over time, whereas a stable symbiotic diversity was maintained when exposed to its native water (Lousada) (Fig. 5.2A top; Table S1B). For *P. perezi,* the variable Day alone affected significantly the skin communities, with tadpoles exposed to both native (Gafanha) and translocated (Lousada) waters showing similar increasing trends of α-diversity over time (Fig. 5.2A bottom; Table S1A). Regarding the gut communities, *P. perezi* did not exhibited any alteration in bacterial α-diversity in neither of the water sources. The two communities were stable across the experiment with tadpoles exposed to their native water (Gafanha) showing a trend for higher α-diversity although not significant. *B. spinosus* registered different responses across sampling days depending of which water tadpoles were exposed to (Fig. 5.3A; Table S1C-D). Specifically, tadpoles exhibited lower gut bacterial diversity at day 0 but significantly increase over days when exposed to the translocated water (Gafanha) whereas when exposed to native water (Lousada) the bacterial diversity was more stable but lower at the end of the experiment (day 28).

Regarding the β-diversity, our results showed that variation in skin and gut bacterial composition was explained by host identity (8% and 5.9% respectively), by water origin within host (5% and 10.3%, respectively), and by days within water and host (8.7% and 10.8%, respectively) (Fig. 5.2B, 5.3B; Table S2). This can also be observed in the NMDS plot where skin bacterial communities are clearly clustered by host species and gut communities by host species and water origin (Fig. 5.2B, 5.3B). Moreover, in both communities (skin and gut) the ellipse of each species when exposed to water from Gafanha are always bigger than the one from individuals exposed to the water from Lousada, indicating that the water from Gafanha is responsible for higher diversity.

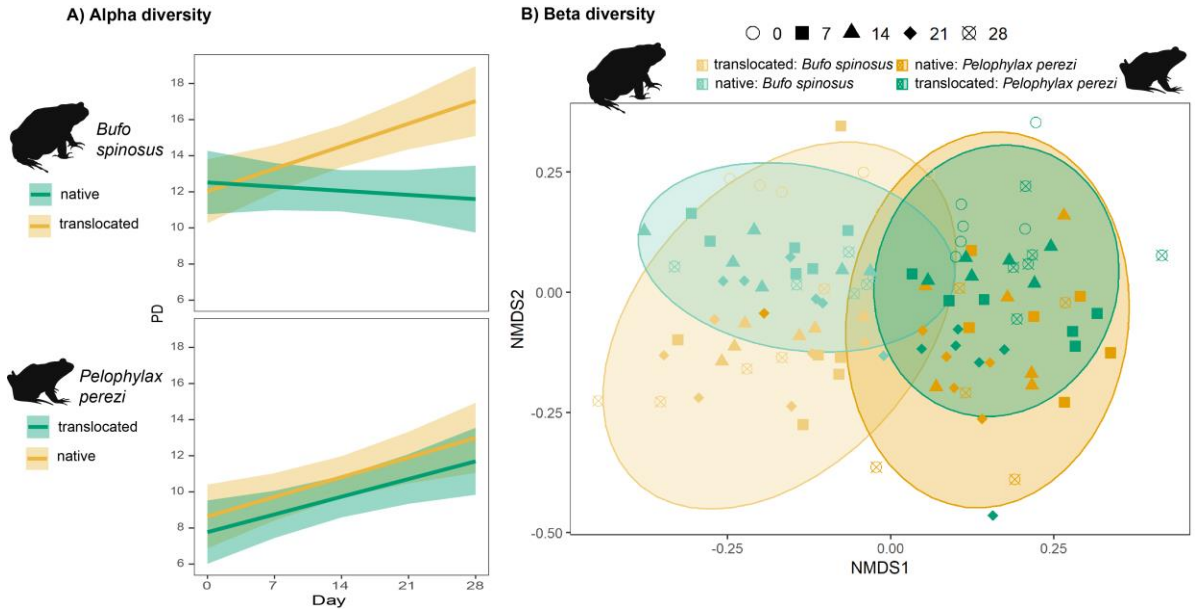

Figure 5. 2. Skin-associated bacterial communities of tadpoles exposed to native and translocated waters sources over time (Gafanha and Lousada): (A) phylogenetic α-diversity detected in each host species, *Bufo spinosus* (top) and *Pelophylax perezi* (bottom); and (B) β-diversity (using unweighted Unifrac distances) detected among tadpoles of each species. Yellow and green color shades represent the sampling locality of the water, ellipses denote the four experimental groups, and symbols indicate sampling days. *P. perezi* tadpoles were collected at Gafanha and *B. spinosus* tadpoles at Lousada, which correspond to their native source waters respectively.

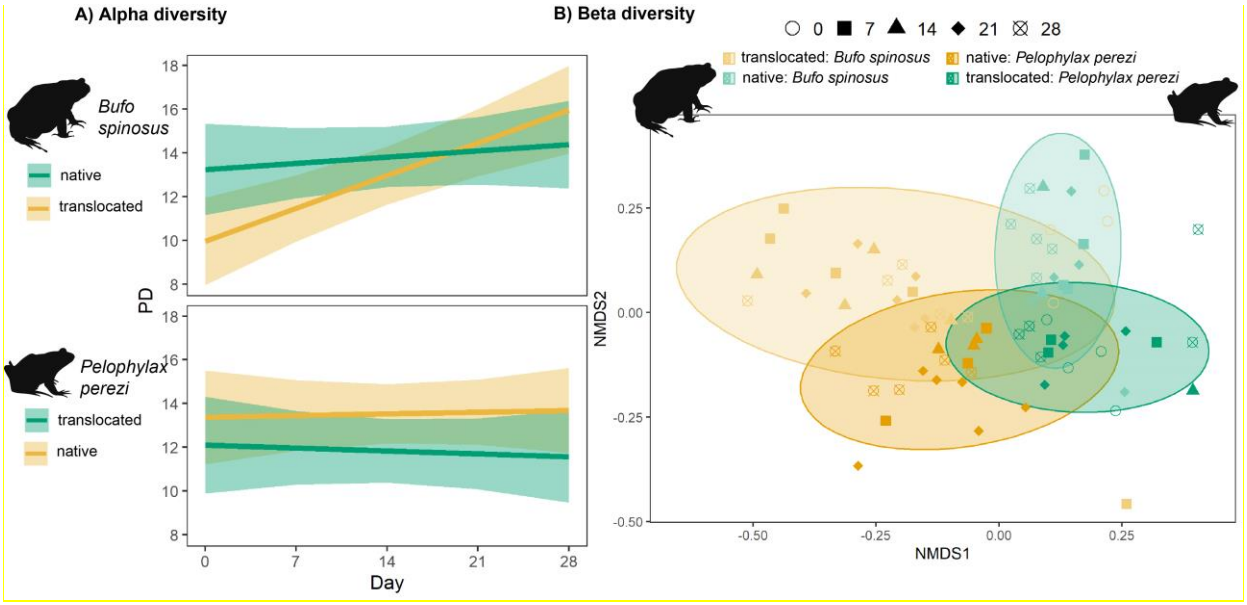

Figure 5. 3. Skin-associated bacterial communities of tadpoles exposed to native and translocated waters sources over time (Gafanha and Lousada): (A) phylogenetic α-diversity detected in each host species, *Bufo spinosus* (top) and *Pelophylax perezi* (bottom); and (B) β-diversity (using unweighted Unifrac distances) detected among tadpoles of each species. Yellow and green color shades represent the sampling locality of the water, ellipses denote the four experimental groups, and symbols indicate sampling days. *P.*

perezi tadpoles were collected at Gafanha and *B. spinosus* tadpoles at Lousada, which correspond to their native source waters respectively.

In terms of the relative abundance of the main taxa (at phylum and class levels), differences were observed between skin and gut communities, and between water and gut bacterial communities, with water and skin being more similar to each other for all experimental groups (Fig. 5.4): for example, Actinobacteria was generally more abundant in the water and the tadpole's skin than in the gut for both species; on the opposite, Planctomycetes was consistently more abundant in the gut of both species; Proteobacteria was the most abundant phylum in the water samples and in the skin communities, while in the gut communities it varied across species, water and days; Firmicutes was consistently more abundant in the gut rather than in the water or the skin; Bacteroidetes was consistently more abundant in skin communities (especially in *B. spinosus*) rather than in water and the gut. At the class level, gut communities of *P. perezi* tadpoles exposed to native water (Gafanha) showed high abundance of Bacilli, this class being one of the most abundant also in the water source (Fig. S2). Contrary, tadpoles exposed to translocated water (Lousada) did not have the same pattern. Similar to what was observed at the Phylum level, also at the class level the abundances of bacterial taxa of water and skin were more similar to each other that to the gut communities in all experimental groups (Fig. 5.5), for example: both species consistently exhibited high abundance of Betaproteobacteria in the skin and water, while the gut communities had higher abundances of Alphaproteobacteria. Planctomycetia was associated with the gut and observed in both species across sampling days and in both Gafanha and Lousada source waters.

The LEfSe analysis identified a total of 19 differential abundant OTUs in the skin and 27 in the gut communities, depending on the species, the water to which they were exposed and the sampling day (Figs. 5.6, 5.7). In the skin communities, the two species had more differentially abundant OTUs at day 0 (Fig. 5.6) and *P. perezi* exposed to translocated water (Lousada). In the gut communities, individuals of both species showed a high number of differential abundant OTUs at day 0 or at the end of experiment (day 28) when exposed to water collected from Lousada (regardless if this was native (in the case of *B. spinosus*) or translocated water (in the case of *P. perezi*); Fig. 5.7). In the two communities (skin and gut) there were differentially abundant OTU's belonging to Proteobacteria, Bacteroidetes, Fusobacteria, Firmicutes, Planctomycetes, Actinobacteria and Verrucomicrobia, the latter being differentially abundant only in the gut community of the tadpoles of *B. spinosus* exposed to native water (Fig. 5.7).

The Proteobacteria group was consistently the most abundant in the skin assemblages but OTU's belonging to different families differed between groups (Fig 5.6). *P. perezi* exposed to native water (Gafanha) exhibited only one significantly abundant taxon, *Nevskia ramosa*. *B. spinosus* also exhibited only one abundant taxon in each water: *Flavobacterium succinicans* when exposed to native (Lousada) water and *Hydrocarboniphaga effusa* when exposed to translocated (Gafanha) water.

In the gut assemblages, tadpoles of *B. spinosus* exposed to native water for four weeks exhibited the same *Rhodobacter* genus as the one found at the beginning of the experiment (day 0), but this was not observed in the tadpoles exposed to the translocated water.

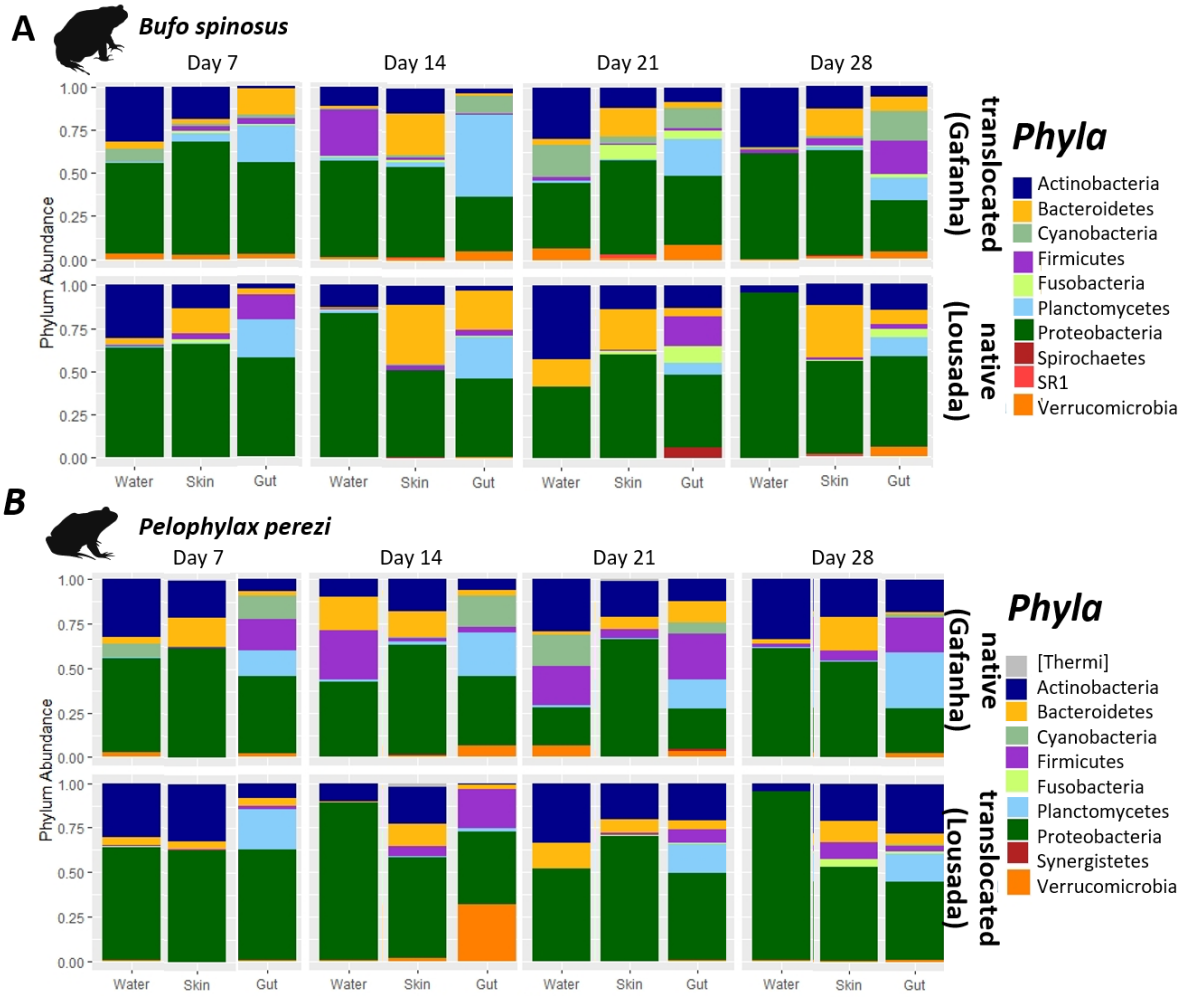

Figure 5. 4. Abundance of the 10 most prevalent bacterial Phyla in the skin and gut communities of (A) *Bufo spinosus* and (B) *Pelophylax perezi* in comparison with those found in the water where tadpoles were reared at each sampling event (Day).

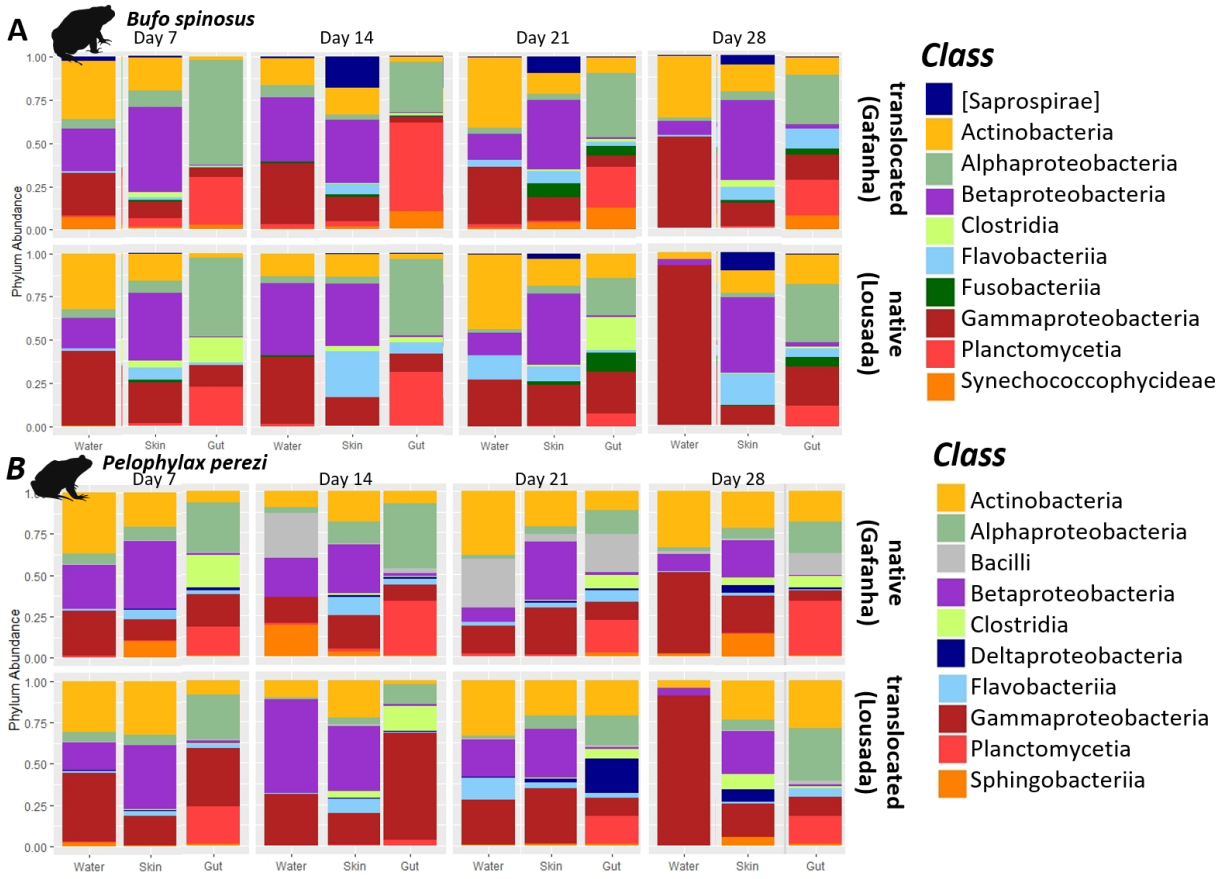

Figure 5. 5. Abundance of the 10 most prevalent bacterial classes in the skin and gut communities of (A) *Bufo spinosus* and (B) *Pelophylax perezi* in comparison with those found in the water where tadpoles were reared at each sampling event (Day).

In terms of total of unique and shared OTUs between groups, we observed that the two species before the experiment had a higher number of unique OTUs and less than 10% of taxa were shared in the skin community whereas the gut exhibited the same patterns but with even lower percentage of shared OTUs (5.4%, Fig. 5.8). At the end of the experiment, each group (host versus water origin) had a higher number of unique OTUs than shared ones and that the patterns varied between skin and gut communities (Fig. 5.9). Each species exposed to water from Gafanha exhibited the higher values of unique OTUs. When comparing the total of unique OTUs that were shared within species or between species we observed that the two species that were maintained at water from Gafanha shared more unique OTUs with each other than with itself when exposed to Lousada water. In the gut, the lower values of shared OTUs were observed within species when exposed to different waters (*B. spinosus* = 23; *P. perezi* = 14).

Following the same patterns observed for the other diversity indexes, tadpoles of *B. spinosus* exhibited a higher number of common OTUs between the beginning of the experiment (day 0) and the end of the experiment exposed to both waters (day 28) indicating that these OTUs may constitute a stable community in this species. This high number was observed for the skin and gut communities when compared with tadpoles of *P. perezi*. When comparing only the core100, we observed that *B. spinosus* carry 9 skin OTUs and 6 gut OTUs in all the individuals *while P. perezi* carry 2 skin OTUs, one that was also observed in the other species and none in the gut (Fig. S4).

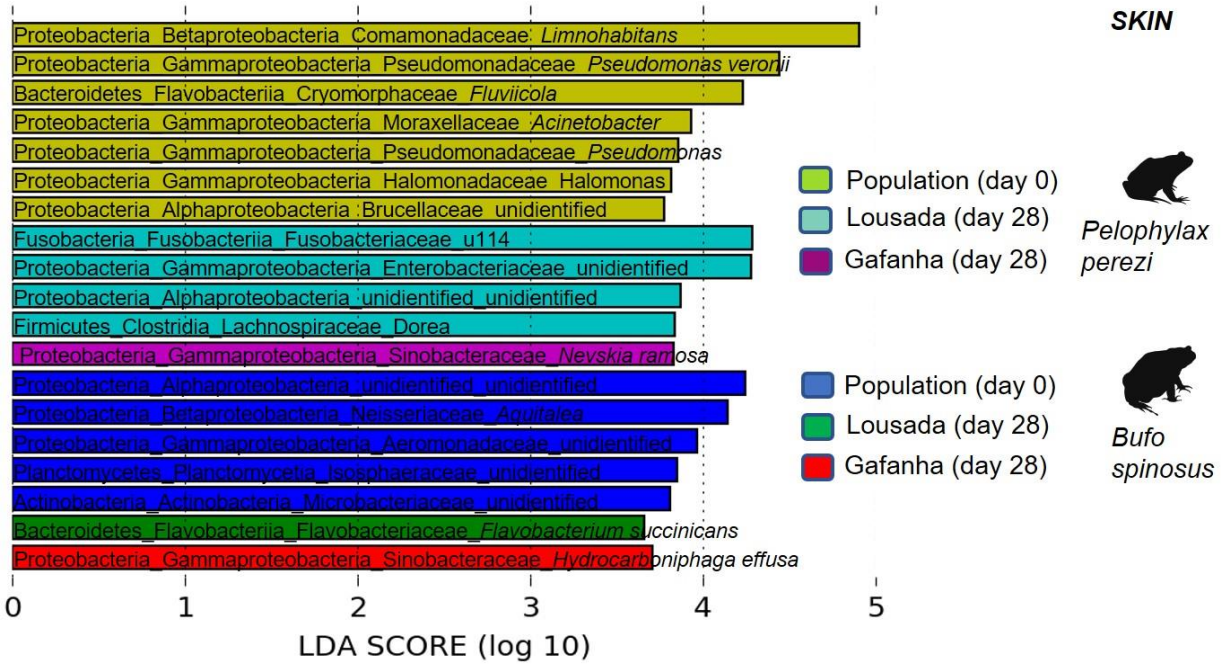

Figure 5. 6. Identification of the skin bacterial OTUs that were significantly more abundant in each experimental group (host x water) at the beginning and end of the experiment. Bars represent the values for significant LDA scores (LDA>2) of each OTU.

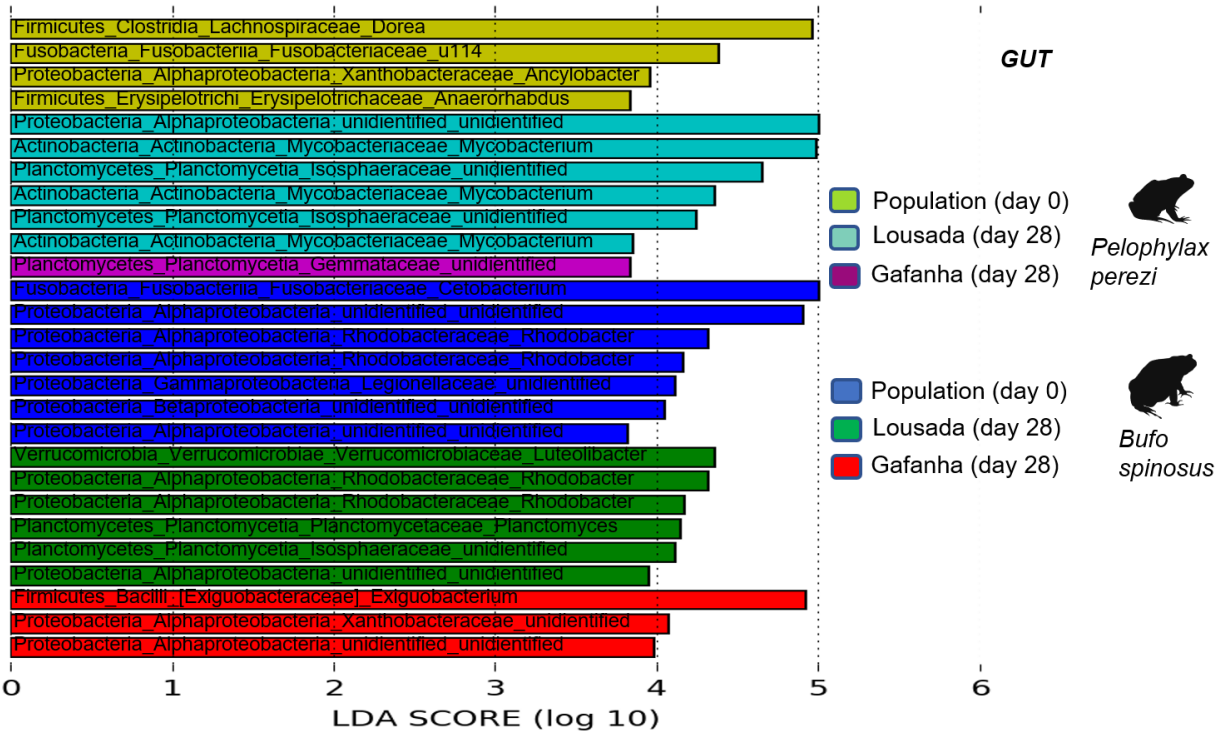

Figure 5. 7. Identification of the gut bacterial OTUs that were significantly more abundant in each experimental group (host x water) at the beginning and end of the experiment. Bars represent the values for significant LDA scores (LDA>2) of each OTU.

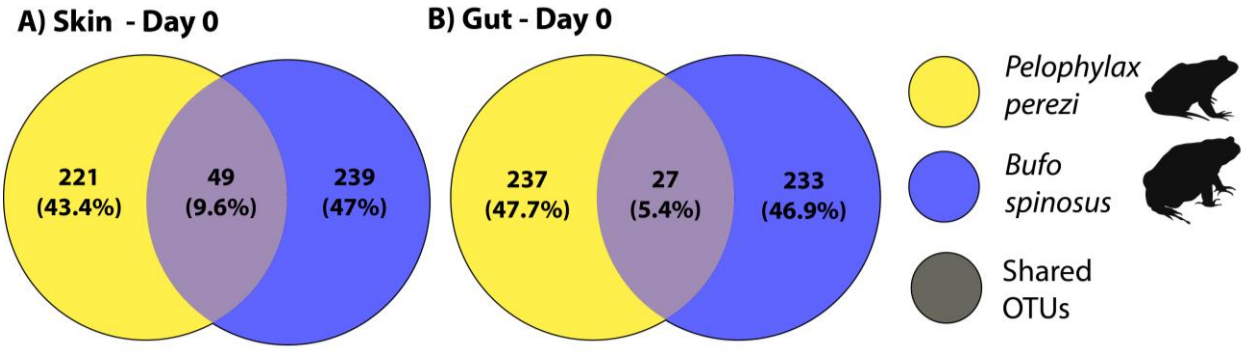

Figure 5. 8. Total of unique and shared bacterial OTUs between tadpoles of *Pelophylax perezi* (yellow) and *Bufo spinosus* (blue) at the time of collection: (A) Skin, (B) Gut

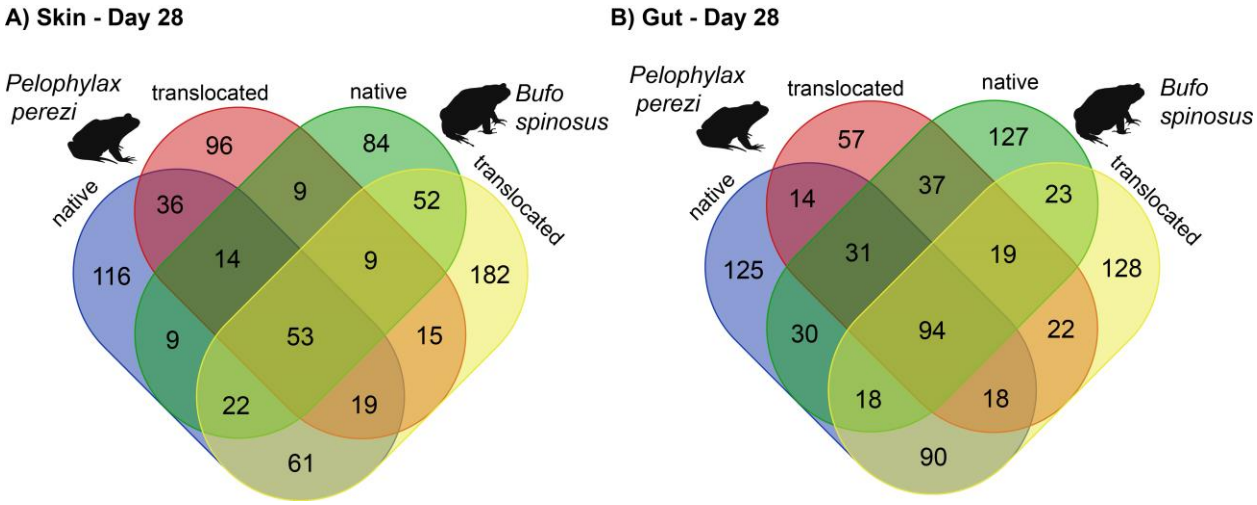

Figure 5. 9. Total of unique and shared bacterial OTUs between tadpoles of *Pelophylax perezi* and *Bufo spinosus* at the end of the experiment (day 28) after being exposed to native and translocated waters: (A) Skin, (B) Gut

DISCUSSION

Several studies have highlighted that the surrounding water environment has a strong influence in the host symbiotic bacterial communities (Jani & Briggs, 2018). However, most of them have focused on natural populations where the individuals are naturally exposed not only to water but to a panoply of other environmental factors. Designing a laboratory experiment where all environmental conditions are better controlled allows to better individualize the effects of different factors such as the host identity and water environment, and to minimize the confounding factors found in natural settings (e.g., climatic and environmental features, presence of other organisms, food scarcity). Moreover, studies on the temporal variation and stability of the microbiomes in non-model organisms are still rare (Harrison et al., 2019).

The present work aimed to characterize the effects of the exposure to a new water environment (as proxy to habitat alteration scenario) in the skin and gut bacterial communities of tadpoles of two amphibian species collected from different sites and maintained in similar controlled laboratory conditions over a four-week period. Our results showed that there is a significant effect of the host species, water origin and experimental days that influence the diversity and composition of skin and gut bacterial communities in one species while for the other the bacterial diversity patterns was influenced only by experimental day.

- **Source waters and experimental conditions**

The water collected at the two water sources (Gafanha and Lousada) had, as expected, different alpha and beta diversities. The bacterial community of the water collected in Gafanha was more diverse (consistently across the four-week experiment), suggesting that the tadpoles of both species exposed to this water have been subjected to a more diverse and richer environmental bacterial pool. The community structure also differed between the two source water bodies, a pattern that was observed across the experimental days, therefore ensuring that the two water bacterial pools to which the tadpoles were exposed throughout the experiment were consistently different.

- **Differences in host symbiotic communities at day 0**

The two host species collected at two different sites had a significantly different initial skin community (Day 0), with tadpoles of *B. spinosus* harboring a richer skin community comparatively to *P. perezi*, despite the higher microbial diversity observed in the natural environment of the latter (i.e., Gafanha). The differences observed in the skin communities of the two species can also be linked to the type of water body. For example, amphibians from permanent lotic waterbodies (like *B. spinosus* here) have been associated with richer skin bacterial communities (Sanchez et al., 2017) due to different water properties (e.g., nutrients availability, organic matter and dissolved oxygen, temperature). On the other side, the higher bacterial diversity observed in Gafanha water can be related to the presence of agricultural fields near the sampling site, with likely run-offs products from the soils leading to more frequent alterations in water composition. Agriculture soils treated with fertilizers or pesticides can exhibit an increase of dissolved organic matter and consequently an increase in the microbial diversity that in turn could affect water environments (Chen et al., 2018; Wilson & Xenopoulos, 2009). Species collected from agricultural areas have been found to carry a symbiotic community with higher functional diversity often associated with responses to pathogenic diseases, pesticides degradation or alterations on diet habits but not necessarily higher taxonomic richness or diversity (Chang et al., 2016; Huang et al., 2018) which was observed in *P. perezi*. The gut communities of the two species did not differ in terms of alpha-diversity but shared few OTUs suggesting that habitat type (including water characteristics and prey availability) is, as expected, influencing the bacterial composition of the two species at the tadpoles' stage (Chang et al., 2016).

- **Differences in bacterial communities across the experiment**

Water environment can affect the symbiotic communities occurring in all host body layers, from the most exposed one such as the skin to a more internal and specific like the gut (Bletz et al., 2016; Kueneman et al., 2014). After assuring the two host species were exposed to the same experimental conditions, we characterized the variation in tadpoles' microbial communities exposed to each water over time. Our main objectives consisted in i) assessing if tadpoles of the two target species exposed to a new environment would experience a shift in their skin and gut symbiotic communities, and ii) comparing the response patterns of each community (skin and gut) in each water.

The differences found between the two hosts at day 0 were also observed throughout the experiment but at this point were influenced by host, habitat (as water source) and sampling day. The two species were able to maintain their distinct skin bacteria community with low overlap, (Fig. 2B, 3B) supporting the role of the host identity as one of the most important factors influencing symbiotic communities in amphibians populations (Bletz et al., 2017b; Ellison et al., 2018). This host effect is normally associated with the genetic background of the host species but also with its ecology/behavior (aquatic vs terrestrial; Bletz et al. 2017a) and physiology (e.g., skin properties that can filter different bacterial taxa; Bletz et al. 2017c), among other traits. Particularly, the skin structure and mucus production and peptides are suggested to have a crucial role in shaping skin bacteria communities while differing among host species (Rollins-Smith et al., 2005; Woodhams et al., 2014). *B. spinosus* adults are known to have a very dry and rugous skin with warts and glands that can produce toxic secretions, while *P. perezi* have a smoother skin. Although frogs and toads have similar skin texture and appearance in their early aquatic life stages, the skin of tadpoles of several bufonid species can produce noxious secretions to deter predators, and possibly influencing its bacterial composition (Regueira et al., 2016). This host effect was less marked in the gut communities. Tadpoles of *P. perezi* were able to maintained a stable gut community diversity across the four-week experiment in both waters suggesting that the gut bacteria of this species may be more stable and resilient to some environmental alterations. However, since all tadpoles were exposed to a new diet that is expected to influence the gut microbiome (Chai et al., 2018; Vences et al., 2016), the lack of more significant differences is unexpected. Once again, *B. spinosus* exhibited a different pattern from *P. perezi*, where tadpoles showed an increase richness across the sampling days when exposed to the translocated water source, indicating that this could result from the host identity (e.g., the gut would be more colonizable than the one of *P. perezi*) and from the water source (e.g., richer

environmental pool). Moreover, tadpoles of *B. spinosus* either from day0 or at each water from day 28 shared more taxa (11%) and also had a richer core100 than *P. perezi* suggesting that it carries OTUs that are probably permanently associated with the host.

B. spinosus maintained consistently a higher skin alpha diversity, and when exposed to translocated water (Gafanha), both skin and gut communities showed an increase in alpha diversity across sampling days, suggesting that the species probably can actively filter more bacteria from the new surrounding environment (Walke et al., 2014b). Since *B. spinosus* tadpoles were exposed in the laboratory to a richer water environment (Gafanha water), it is possible that this new environment acted as a source pool of new bacteria able to colonize toad skin and gut and that were not previously occurring the its native environment.

Similarly, the entire community composition associated to the skin of *B. spinosus* tadpoles exposed to native water (Lousada) only partially overlapped with the community associated to tadpoles exposed to the richer translocated water (Gafanha) supporting the hypothesis that these differences result from the acquisition of new taxa from the translocated source water (Fig. 2B). Our results suggest that, at least for *B. spinosus*, tadpoles exposed to native water (Lousada) maintained stable skin-associated bacteria communities over time, while tadpoles exposed to a richer environment (Gafanha water) acquired a richer symbiotic community. This supports the hypothesis that water source is a remarkable variable affecting symbiotic bacteria in tadpoles of this species as been also observed in other amphibians in captivity and natural conditions (Harrison et al., 2019; Kueneman et al., 2014). Looking at *P. perezi*, tadpoles exhibited similar skin bacterial communities when exposed to both native and translocated water indicating that for this species, water origin did not differently affect the skin communities. This outcome could be related to the fact that this species was originated from a richer water environment (Gafanha) as mentioned above, and thus could already harbor more permanent and stable bacterial communities with little opening for new bacteria to colonize. This hypothesis is supported by the high overlap in the skin community composition between the two source waters (Fig. 2B) indicating that the new taxa that were perhaps colonizing the skin were similar between water sources. On the contrary, this was not so marked in the gut bacterial communities where lower overlap in bacterial composition was observed indicating here that both host identity as well as water origin significantly affected the gut communities. However, since in terms of alpha-diversity, the gut communities of tadpoles exposed to each water were stable and similar, it is suggesting the gut microbiota of *P. perezi* may be more resilient to changes, may change at slower rates that may surpass the four-week period tested here or that the number of new bacteria received from

the new water source was able to colonized the gut by replacing previous taxa that were occurring in tadpoles at day 0.

As expected, gut communities were the most different from water and skin communities, exhibiting high relative abundance of Planctomycetes in both hosts, a common phylum occurring within the gut of tadpoles, the gut of several animals and also in water and soil environments (Bletz et al., 2017; Vences et al., 2016).

- **Differences in communities at the end of the experiment**

The relative abundance levels of many taxa were a good indicator of experimental groups being particularly different between skin and gut communities. Bacterial assemblages of each species varied depending to which water were exposed, and gut-associated communities were more distinct from water and skin. This was in line with lefse analysis that indicated that each species presented different indicative OTUs depending on the water source and these differences were especially marked in the gut community. Although it seems the species were able to receive bacteria from the surrounding water environment during the experiment (especially *B. spinosus* from translocated water), the two tadpole communities (*B. spinosus* and *P. perezi*) at the beginning of the experiment (Day 0) had more distinctively abundant OTUs indicating that natural conditions were responsible for high diversity of each community although it is impossible to distinguish among all the environmental variables associated to each sampling site and not addressed here. On the other hand, across weeks, the new collected water at each site exhibited a decrease in phylogenetic diversity that would result in less diverse water communities to which the tadpoles were exposed. Nevertheless, this did not cause a decrease on the tadpoles' bacterial diversity levels but the opposite. At the same time, despite using the same water sources and laboratorial conditions for the two species, at the end of the experiment, each group (species versus water) were able to maintained its skin bacterial signature by carrying distinct communities (beta diversity) and a high number of unique OTUs comparing with the number of shared ones. This indicates that both amphibian species and water source were able to significant influence the skin and gut communities. In addition, each species exposed to the Gafanha water was observed to share more skin OTUs with the other species than with its own species exposed to Lousada water, indicating that these OTUs were probably acquired from the water to which they were exposed to. The same was observed for the gut communities, although in this case it includes also tadpoles exposed to Lousada water. The bacterial composition of the two species across the experiment differed between them and were clearly different depending on the water to which they were exposed, indicating that the gut of tadpoles were received different bacteria in

response to different water sources (Muletz Wolz et al., 2018) and more important that water effect was stronger than the host effect in gut communities composition. This result is corroborated by the LEfSe analysis indicating that the tadpole's gut harbored significantly abundant OTUs in each water source at the end of the experiment. While tadpoles exposed to water with richer alpha-diversity (Gafanha) showed a trend for increasing their alpha diversity, beta composition and the amount of unique OTUs, they also exhibited lower number of significantly highly abundant OTUs, possibly indicating a trade-off between maintaining and receiving more OTUs from a richer environment, and maintaining stable abundance levels of the most abundant taxa. In fact, tadpoles from both species exposed to water with poorer alpha diversity (Lousada) harbored more differently abundant OTUs (either in the skin or in the gut) and less unique OTUs that may indicate that some OTUs were able to increase in abundance and fill the available space in a less diverse water environment.

- **Possible confounding effects of diet and captivity**

Habitat and diet enrichment have been found to significantly affect amphibians' microbiome while in captivity, since new environment conditions may alter selective pressures on the symbionts (Antwis et al., 2014; Harrison et al., 2017). Most of the studies evaluating the effects of captivity on the microbiome were developed maintaining the hosts in captive conditions for long periods (Bataille et al., 2016; Flechas etal., 2017; Harrison et al., 2019; Sabino-Pinto et al., 2016). Since the conditions of the captive environment are more homogeneous than the ones found in the wild, captive individuals are expected to exhibit poorer symbiotic communities (Bates et al., 2019; Sabino-Pinto et al., 2016), although in some instances, some species exhibited an increase in phylogenetic diversity when maintained for short periods (Becker et al., 2014; Hernández-Gómez et al., 2019). Therefore, the loss of bacterial richness has been associated with longer captivity periods (Kueneman et al., 2016). In this experiment, of short temporal duration and in which tadpoles were exposed to freshly renewed water every week, it was observed an increase of bacterial phylogenetic diversity in both analyzed species. This might be due to the fact that tadpoles were exposed to new transient bacteria each week (Harrison et al., 2017; Loudon et al., 2014). Moreover, during the experiment, tadpoles were not isolated and the contact between individuals might have favored the transfer of microbes among hosts (Banning et al., 2008). Finally, we observed different responses and community composition in the two analyzed species, while a stronger overlap was supposed to be observed if the effect of captivity was significant (Fig. 2B, 3B) and therefore we can assume that the effect of captivity was small and similar between the two species due to the same exposing conditions.

Diet is also known to affect both skin and gut bacterial composition (Antwis et al., 2014; Vences et al., 2016). Here, the two species were fed with the same commercial dietary supplement and therefore the effects of food type on tadpoles' gut microbiota between species were expected to be similar and limited across the experiment. Nonetheless, we expected that the contact with a new food source at the beginning of the experiment could led to a change in gut composition for both species. In terms of alpha-diversity, only the gut bacterial communities of *B. spinosus* exposed to translocated water changed, suggesting that host identity and water source are the main predictors and that the exposure to a new diet had only limited effect. On the other hand, the tadpoles of *P. perezi* exhibited a stable phylogenetic diversity in the gut-associated community, independently of the water to which they were exposed, suggesting a very low influence of the new diet on its gut bacteria. Despite the high level of differentiation identified in both the skin and gut communities of the two species, at the end of the experiment, both species exhibited differential abundant taxa that might have resulted from the captivity period. It is the case of the tadpoles of *P. perezi* and *B. spinosus* exposed to native water that had higher levels of *Nevskia ramosa* and *Flavobacterium succinicans, respectively*. These two bacterial species have been observed to be abundant in captive amphibians (Hernández-Gómez et al., 2019), and for this reason remains difficult to discern between the captivity or the water source effect.

CONCLUSIONS

This experiment shows that both host identity and water source greatly influence the bacterial communities in the tadpole stage, and that the effects vary with the host species and the bacterial community analyzed (skin or gut) while the captivity and alteration of the diet is expected to also have some influence on the bacterial communities but similar between species. The two species maintained their distinct microbial composition throughout the experiment, and these differences were observed in both skin and gut communities (specially in terms of beta diversity and unique OTUs). The bacterial communities of *Bufo spinosu*s were more affected by water treatments than *Pelophylax perezi*, and were found to greatly vary across time when exposed to a richer water source than its native environment. On the other hand, the bacterial communities of *P. perezi* (despite showing a poorer bacterial community when collected) were not as greatly affected by water exposition like *B. spinosus*, showing few differences in terms of β-diversity and total of unique OTUs, and an increase in α-diversity comparable in both waters. We confirmed the role of the water environment in shaping the skin and gut microbiota of tadpoles from different amphibian

species, with each group carrying different numbers of unique OTUs and OTUs with significantly different abundances by the end of the experiment. Using a laboratorial setting, considerably improves the capacity isolate the role of each factor: host species versus water source while excluding the effects of other environmental factors that would be present in a field experiment (climate, diet). Future work should try to determine if there are specific exchanges between some bacterial taxa (e.g., substitution, lose or gain of specific OTUs), assess which are their physiological roles within the host and test if there is an association with the specific water characteristics and bacterial pool.

AUTHOR CONTRIBUTIONS

BS and AC design the laboratorial experiment; BS performed the fieldwork, laboratorial experiment and sample collection; BS, FMSM and JSB analyzed the data and prepared the final data plots; BS and AC wrote the first draft of the manuscript; all authors revised and contributed to the final versions of the manuscript.

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CHAPTER VI

General Discussion and Conclusions

Combining experiments from controlled laboratory environments and natural settings, and targeting different questions ranging from the identity and ecology of anurans to the development of amphibian larvae, I contributed to the development of the use of this emerging discipline in non-model amphibian organisms. This thesis aimed at gathering data of amphibian microbiome including host species from Portugal and Madagascar, across developmental stages and from different habitat types. We explored bacterial communities from amphibians either from natural populations and using experimental settings. This work provided insights about the importance of assessing abiotic factors such as water pH levels in shaping the skin bacterial communities of a native Madagascar species (**Chapter II**); provides the first bacterial characterization on the invasive toad in Madagascar (**Chapter III**); shows that the bacterial communities from co-occurring amphibian species are different across different metamorphosis stages (**Chapter III**); and provides evidence for a distinct effect of the water environment on the skin and gut communities of tadpoles depending on the host species (**Chapter IV**). Moreover, both skin and gut communities were characterized in order to understand how the two communities respond to the same factors.

1. Main microbiota trends observed in this work

- **Skin Microbiome**

We observed that the skin bacteria was affected by biotic and abiotic environmental factors as well as host-associated parameters being in accordance with previous works (Jiménez & Sommer, 2017) and that in some cases, alterations in the skin microbiota were accompanied by shifts in taxa occurrence with anti-fungal properties that ultimately may affect the efficacy of the host immune system (Becker & Harris, 2010).

Host species

Host identity was found to be a significant influencing factor of symbiotic community composition in our study species (Jiménez & Sommer, 2017), with the exception of the two amphibian species analyzed in **Chapter II**. Differently from what it has been observed in several published studies (e.g., Kueneman et al. 2014, Bletz et al. 2017b), and also in most of the studies presented in this thesis (**Chapter III**, **Chapter IV**, **Chapter V**), the bacterial communities of the two species occurring in different microhabitats (**Chapter II**) lacked significant differences in both alpha and beta diversity metrics. This has been previously observed in some other species and the lack of differentiation may indicate that the two species are selecting a similar pool of bacterial taxa from the common environment (Muletz Wolz et al., 2018) which was observed across the three studied habitats supporting this hypothesis.

While previous studies reported lower microbiota diversity in the skin of an invasive species in comparison to the skin of sympatric species (Christian et al., 2018), here we found evidences for the opposite pattern (**Chapter III**). The two analyzed species carried significant different skin microbiota, with the invasive Asian toad carrying a richer and more diverse skin community than the native frog, a condition that could confer adaptive benefits (such as higher fitness and adaptability) to the invasive host as it has been found in other organisms such as insects, plants or seaweeds (Aires et al., 2016; Cheng et al., 2018; Coats & Rumpho, 2014). The high richness and diversity have been linked to amphibian-specific skin properties and differential selection of environmental bacteria or ecological niches (Bletz et al., 2017) but further analysis of the chemical properties of the toad skin is necessary to understand how its symbiotic diversity is selected and if the microbiota can affect the host's invasive potential.

The effect of host identity on the skin-associated microbiota was analyzed also in **Chapter IV**, where we demonstrated that three co-occurring species from a small ephemeral pond maintained a different composition of the skin microbiota, highlighting a strong host filtering effect (Belden et al., 2015). The bacterial communities of the two anuran species were markedly different from the skin-associated bacteria of the urodele species, evidencing some phylosymbiosis signal, as already identified in previous studies (Bletz et al., 2017b; Ellison et al., 2018). Urodele species carry a richer and more diverse skin community (Ellison et al., 2018), but also show less differentiation from the bacterial pool found in the surrounding water environment, suggesting that the filtering process may be different between amphibian orders and it can possibly be linked to skin chemistry and properties (e.g. texture, secretions). Different host associated skin microbiota

may also affect the prevalence of probiotic bacteria, as observed in the skin of *P. mascareniensis* (**Chapter III**).

Host development

While it is known that skin microbiota shifts across amphibian developmental stages (Kohl et al., 2013), and especially between pre and post-metamorphic stages (Jiménez et al., 2019; Kueneman et al., 2014), little is known on the microbiome changes that occur in smaller time-frames during metamorphosis (Prest et al., 2018). Moreover, it is still not clear whether those changes in the skin microbiome follow common trends in amphibian species despite their ecological and physiological differences. We provided a full characterization across four developmental stages during the metamorphosis of two anurans and one urodele species restricted to a single temporary pond to understand if the metamorphosis process has a similar influence on the microbiome of each host (**Chapter IV**). Overall, we found similar patterns of microbiome variation in anurans, with major differences arising when comparing pre- and post-metamorphic stages, while for the urodele species' skin microbiota, this distinction was not so strong. Since the metamorphosis process in known to be accompanied by drastic structural and immunological changes including the reduction of antimicrobial peptides production (Faszewski et al., 2008; Rollins-Smith, 1998; Rollins-Smith et al., 2011), this may indicate that anuran' aquatic stages may undergo less drastic alterations in the skin properties than the urodele species. Previous studies either focused on a single species (Jiménez et al., 2019; Kohl et al., 2013; Prest et al., 2018; Sanchez et al., 2017), or compare multiple species sampled in different years (Ellison et al., 2018), or different sites (Jiménez et al., 2019; Prest et al., 2018), therefore the observed patterns could be partly influenced by spatial or temporal factors. In **Chapter IV**, while comparing three syntopic species, we found that each species has unique microbial community richness and diversity levels during the metamorphosis process, with the urodele species showing the most distinctive responses.

Habitat type

While habitat type and habitat alterations are expected to affect the host-associated bacterial communities (Chang et al., 2016; Hughey et al., 2017), we did not find evidences for that in our **Chapters II** and **III**. The absence of habitat effects in **Chapter II** was unexpected but might be related with sample size, individual home range, or the level of habitat differentiation that may not be enough to translate into microbiome changes or that the habitat effects from the more anthropized habitats may be already extended into the more pristine areas due to the close

proximity. Both *P. mascareniensis* and *M. betsileanus* are common species but, while the first often occupy anthropized habitats, *M. betsileanus* has a preference for less degraded habitats and the occurrence of slow-moving streams. Both species were found across a habitat gradient in Andasibe (central eastern Madagascar) but the clear low abundance of *M. betsileanus* in rice fields and of *P. mascareniensis* in forest sites, indicate that although they have a preference for specific habitats, they can occasionally occur in less suitable ones and this seemed be reflected in the absence of major alterations in the skin microbiota. The reduced phylogenetic diversity of the skin-associated bacterial community observed in *P. mascareniensis* individuals occurring in a pond with basic water pH, was accompanied by a low number of unique taxa. Interestingly, this condition was associated with a notable increased in the proportion of anti-*Bd* taxa, indicating that water pH could have an some influence in modulating skin defenses in amphibians and suggesting that population assessments of amphibian's microbial communities across different sites or habitat should take into account water characteristics (Costa et al., 2016). The influence of water pH in shaping amphibian microbiome and skin properties linked to the immune system of the host (such as the production of AMPs) has been previously detected in post-metamorphic individuals, where the authors observed an increased production of AMPs that have important functions against skin pathogens (Krynak et al., 2015).

Our hypothesis that the toad's microbiota would be changing across its expansion rate (**Chapter III**) was not met, but the absence of the habitat signal may be associated to the limited differences of the surveyed sites. Despite these limited habitat influence, both amphibians were able to maintain their specific microbial signature that was observed across the three sites supporting the hypothesis that the two species carry different microbiomes. The toad' expansion in Toamasina has been followed since its early stages, and the three sampling sites were selected to cover the species expansion from the introduction site (site A) (see Fig. 1 from **Chapter III**). The trend of increasing alpha diversity (from site A to C) may indicate that the toad may be carrying a core microbiome, which is stable and more diverse, and that at the same time is receiving new bacteria across its expansion range. Ultimately, it is likely that the highly anthropogenized environment of Toamasina (toads were collected in villages, livestock pastures and rice fields) restrict the environmental bacterial diversity and availability and consequently, the host symbiotic communities. This was confirmed by the finding that the species analyzed in **Chapter III** exhibited a lower alpha diversity than the average values found for other Malagasy amphibians sampled in native and more pristine habitats (Bletz et al., 2017).

Contrary to **Chapters II** and **III**, in **Chapter V**, we confirm that the exposure of tadpoles to new water sources (used as a surrogate for habitat alteration changes) can quickly affect tadpoles' microbiome and that the habitat effect is different for each species. These data suggest that tadpoles can be used as biological indicators to assess the effects of variations on the surrounding water environment and its effects of the host symbiotic communities and this stage can be particularly useful in comparison with adult stages (most common study subjects) since tadpoles live permanently in the same water body and therefore are continuously exposed to water changes,

- **Gut Microbiome**

Collecting samples for gut microbial analyses has the big disadvantage of requiring the sacrifice of the animal. This brings obvious ethical constraints or limitation to the sampling design, particularly regarding endangered species or longitudinal experiments where the same individual need to be sampled several times. This is one of the reasons why gut microbial communities are far less studied than skin microbiota in amphibians, and why datasets with gut microbial data are usually less robust. A recent study indicate that faeces may not be the best surrogate, but cloacal swabs seem to perform reasonably well, at least in terms of reflecting the bacterial diversity from the large intestine (Zhou et al., 2020). However, cloacal sampling can be challenging (e.g., due to the reduced size of animals). To reduce the negative impacts of sampling natural populations, the collection of gut contents was restricted to the invasive toad in Madagascar, to the abundant co-occurring native species *P. mascareniensis* (**Chapter III**; in this case the sampling of the gut was reduced to only four individuals per site); to five common species found in Portugal for which we decided to target the early (aquatic) life stages and few individuals recently metamorphosized (**Chapter IV, Chapter V**). In my thesis, we observed that the gut microbiome is less affected by environmental variables when compared to the skin's. Observed variation among species and experimental groups was mostly found in terms of abundance levels and in the number of core and unique taxa, rather than alpha and beta diversity.

Host identity

The lack of differences in the gut microbiota in **Chapter III** was previously shown in other species (Huang et al., 2018), although in tadpoles, species identity seems to influence the gut community (Vences et al., 2016). This result may be due to the low sampling size and consequently insufficient statistical power that possibly prevented the identification of differences in alpha and beta metrics but is also probable that the host effect could be neglected in favor of other factors

such as diet and habitat parameters (described below). Despite this, other diversity metrics (differential taxa abundance and percentage of unique OTUs) provide some insights on the gut microbiota differences likely resulting from the ecology of the native and invasive species (e.g., diet, microhabitat preference, body size, physiology) (Vences et al., 2016). However, research studies on adult gut microbiome in amphibians are still lacking (Zhou et al., 2020).

Similarly, to **Chapter III**, the differences in gut microbiota identified between host species from **Chapter IV** were mainly observed in terms of abundance levels and uniqueness of taxa. The absence of more marked differences between anurans and urodele from the same pond may reflect the lack of prey diversity in the water body since they are expected to favor different diets (Chang et al., 2016) or associated to the close synthopy of the three species, favoring microbiota transference.

In **Chapter V**, we observed that tadpoles from different anuran species were able to maintain their distinct gut microbial signature despite being feed the same diet and being exposed to the same water sources. These results are in accordance with previous works (Vences et al., 2016; Warne et al., 2017) and support the hypothesis that the gut community of tadpoles is greatly influenced by host identity (reflecting either genetics, physiology and ecology differences), similarly to what it has been observed for the skin microbiota in adult stages (Warbe et al. 2017, Belden et al. 2015).

Host development

The effect of host development was assessed in **Chapter IV**. Similarly, to what it has been observed for host identity, only taxa abundance and the presence of unique taxa were good indicators of shifts in the gut microbiota. The first two selected aquatic stages were overall more similar among them than with the two later stages and this trend was more marked in *Epidalea calamita*. This pattern has been previously observed in another toad species indicating that can be associated with the increase of intestine length until the early-metamorph followed by a decrease in the two later stages (Chai et al., 2018). The absence of differences in alpha and beta diversity metrics was not expected considering the major changes in gut morphology and physiology occur during metamorphosis that are known to affect bacterial communities (Kohl et al., 2013).

Habitat type

Although previous studies have found that habitat type greatly influence the gut microbiota of amphibians (Chang et al., 2016; Huang et al., 2018), our results indicate it may not always be the

case especially when comparing sites with similar anthropogenic influence (**Chapter III**). Similar to what it has been observed in the skin, no differences were found between the gut communities of the Asian toad and the native frog across the three sampling sites (**Chapter III**) and as for the skin, it can be related with to the highly anthropized and homogeneous habitat in which the two species were sampled, characterized by similar prey availability, or can be the result of the low sample size.

The influence of water source in shaping gut-associated communities was identified in **Chapter IV**, where tadpoles from two species exhibit markedly different gut communities when exposed to different water sources, confirming that the exposition to a new bacterial pool can influence the gut microbiota, in accordance with previous studies of natural populations (Bletz et al., 2016; Harrison et al., 2019). However, it is known that the original environmental pool to which the individual was previous exposed also may have later influences in the bacterial changes either by limiting available niches or by competition with foreigner taxa.

2. Challenges and future work

The study of symbiotic communities is an emerging field of research, and it is still facing many challenges. One of the major challenges is determined by the dynamic and complex nature of these communities, which often difficult the analytical and laboratory procedures. The lack of methodological standardization, together with the rapid growth of available data, is hampering comparisons between different studies and limit inferences at broader scales. In addition, reference databases, particularly for non-model organisms, although they are rapidly growing, remain quite incomplete while collection enough samples from natural populations is sometimes not easy. This was certainly a problem for the analyses presented in **Chapter II** and **Chapter III** where the sampling of Malagasy species reveled to be a challenge due to the low population size of native species contrasting with the high abundant invasive toad. The reduced number of samples probably hindered the identification of differences in bacterial patterns. On the other side, the functional analysis performed in **Chapter III** enabled a preliminary inference of the bacteria functional composition of the native and invasive species and revealed significant differences useful to complement the interpretation of the case study.

In spite of many challenges and limitations, the potential applications on microbial research are numerous. The surrounding habitat can greatly impact microbial communities, and this property may be explored for ecosystem monitoring (Antwis et al., 2017). Given the link between a healthy

microbiome and the fitness of the host, it is crucial when evaluating the impact of human activities on amphibian biodiversity to also take into account the effect of the environmental bacterial pool. Here, we only assessed the effects of bacterial pools resulted from different water sources (**Chapter V**) and water pH (**Chapter II**), but other environmental parameters such as climate, water characteristics, soil type and biotic factors like co-occurring species, density and prey availability, among others, can affect microbiomes and should also be considered. The impact of invasive species on native microbiomes is a recent line of research that may become relevant to understand pathogen transmission and disease outbreaks. In this work, we lack the characterization of the skin and gut bacterial communities of the invasive toad from its native habitat in Asia. The availability of this information will enable to characterize the core bacterial community of the toad and therefore assess its changes during the invasion. In addition, despite several efforts to control the toad invasion in Madagascar, its expansion continues, and there is the risk of soon reaching more pristine areas, therefore the analysis of individuals from different habitats would help to understand how stable is the toad microbiome and to explore the response of the microbiome to habitat alterations.

This work showed that controlled-environment experiments are useful to evaluate abiotic effects on microbiome shifts. Experimental assessments should be coupled with the study of natural populations to enable a better characterization of the individual effects of each factor. In experimental assessment, the use of tadpoles offers a good compromise, giving the possibility to house more easily more individuals and replicas and the possibility to follow the bacterial shifts since host early stages. The analysis of different microbial communities (e.g., skin and gut) in the same study is still rare (Colston & Jackson, 2016; Walker et al., 2020) and should be more often addressed due to the different response that these communities can have to the same factor (as observed in our **Chapter V**).

Microbial research has been focused mainly on the study of bacterial taxa, but the “Microbiome” includes several other organisms that need to be more widely explored to shed some light on microbial networks and to fully understand its role in shaping the response of the host to changes in the environment. Particularly, the amphibian associated fungal communities were shown to reflect alterations in the surrounding habitat of the host and confer some protection against *Bd* (Kearns et al., 2017).

3. Concluding remarks

Overall, the results of this dissertation support the strong host-filtering factor found in numerous previous studies, especially in the skin communities. Our results show the effect of habitat alteration (through water source manipulation or pH) in shaping the microbiome of amphibians at early stages, being in line with previous findings for adult stages. Gut microbiome was found to be generally less variable than the microbiome of the skin, especially in terms of taxonomic richness. While significant changes in host skin and gut microbiota were observed in our study species, the effects and relevance that these alterations may have in the health and fitness of the host remains unclear.

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Appendices

Appendix A - Supplementary material of Chapter II

Exploring the composition of amphibians' skin bacteria from sub-pristine and human-impacted habitats in eastern Madagascar

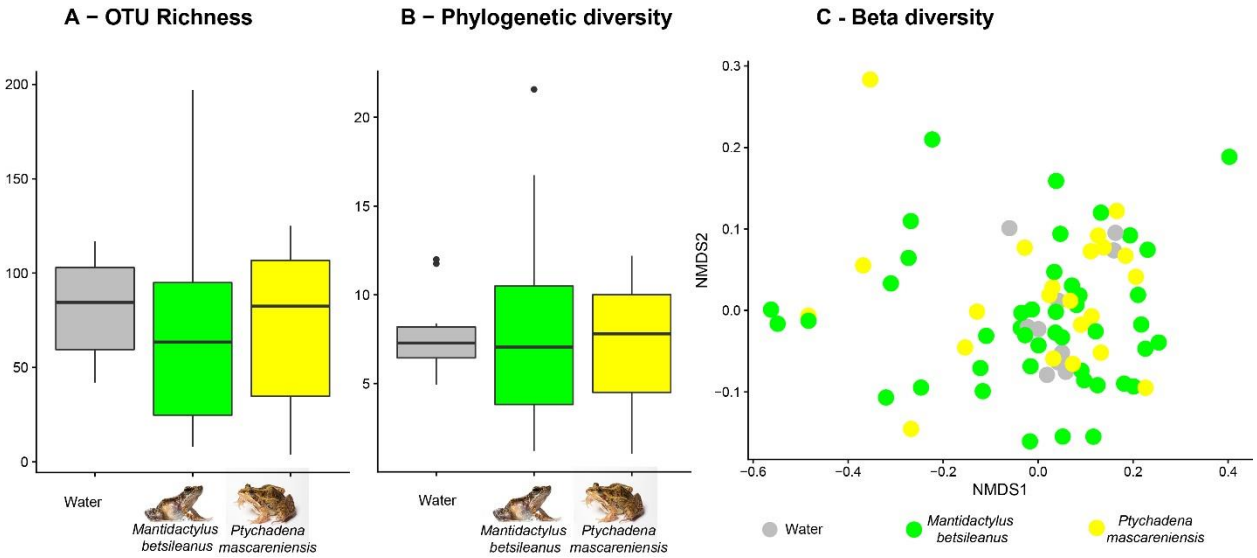


Figure S1 - Alpha and Beta diversity. Alpha diversity is represented as A) OTU richness and B) Phylogenetic diversity and Beta Diversity (C) of skin bacterial from individuals of *Mantidactylus betsileanus*, *Ptychadena mascareniensis*, and water samples combined all samples from the three habitats.

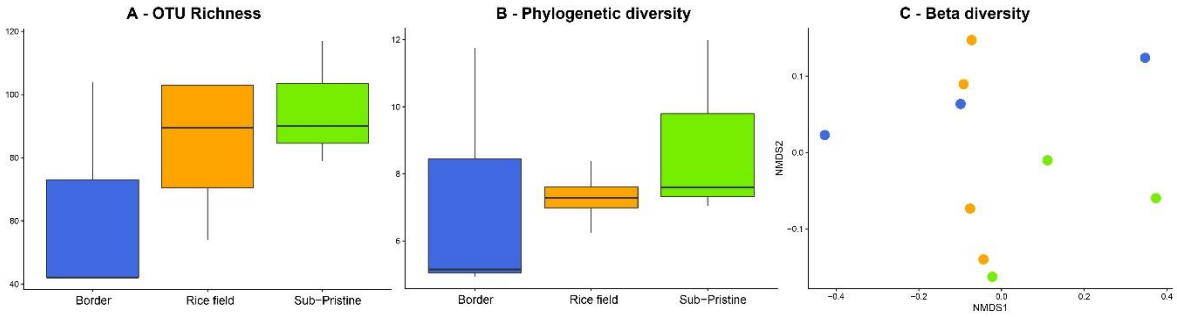


Figure S2 - Alpha and Beta diversity. Alpha diversity is represented as A) OTU richness and B) Phylogenetic diversity and Beta Diversity (C) of water samples across habitats.

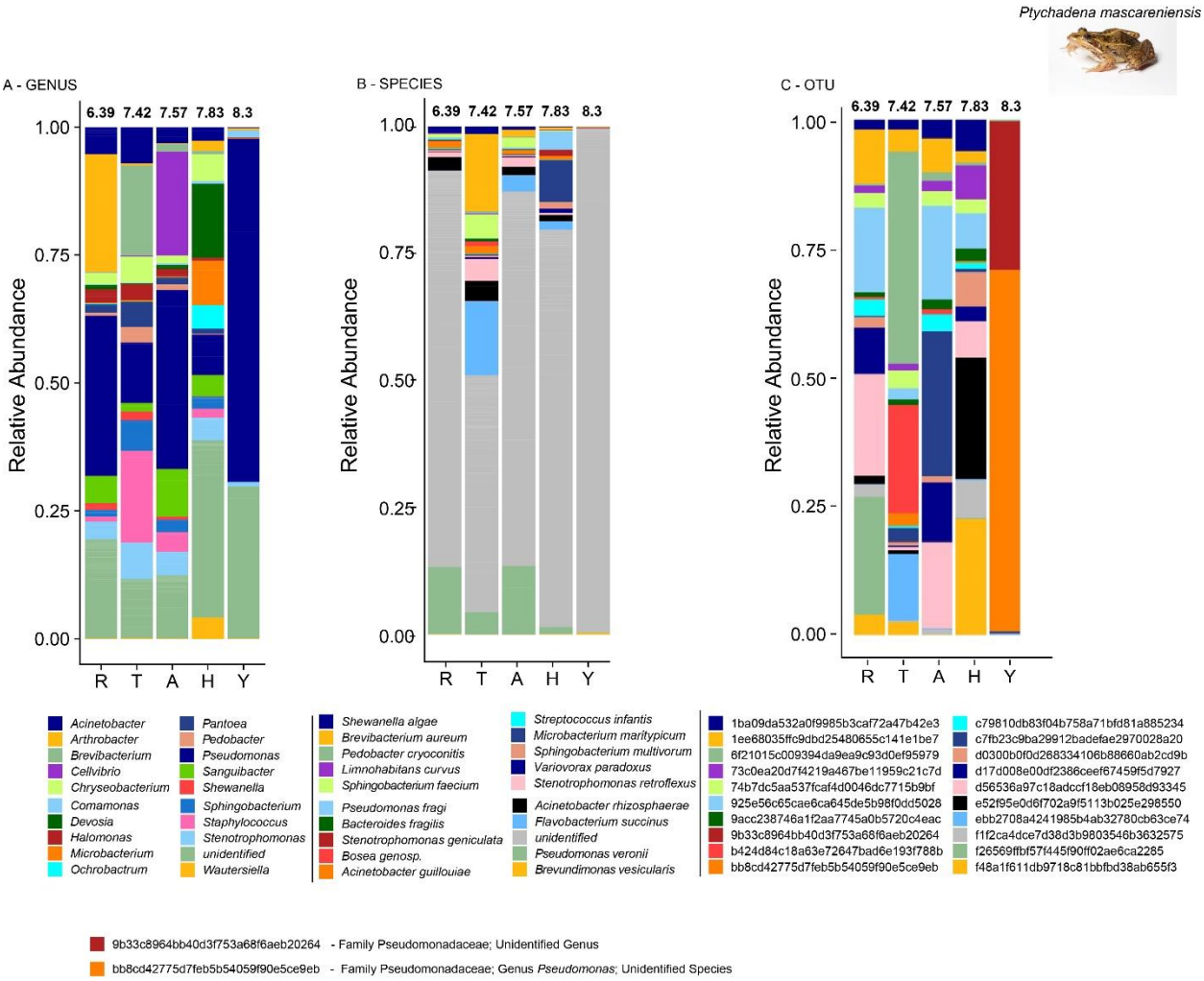


Figure S3 - Relative abundance of taxa at different taxonomic levels of skin-associated bacteria in sampled individuals of Ptychadena mascareniensis from five rice field sites with increasing water pH: A) Genus; B) Species; C) OTU

Table S1 – Table with main test statistics from Alpha diversity from all the datasets. Statistically significant P-values are in **bold** and underlined.

Alpha Diversity Skin bacteria	**Factor**	**Lmer model Type II Wald X^2**	**Degrees of freedom**	**Pr(X^2)**
Dataset A Richness (sOTUs)	Species	2.8142	2	0.2449
	Habitat	0.5793	2	0.7485
	Species * Habitat	2.9066	4	0.5736
Dataset A Faith's phylogenetic diversity (PD)	Species	1.4496	2	0.4844
	Habitat	0.8049	2	0.6687
	Species * Habitat	1.4158	4	0.8414
Alpha Diversity Skin bacteria	**Factor**	**Kruskal-Wallis Rank sum test X^2**	**Degrees of freedom**	**Pr(X^2)**
Dataset B – *P. mascareniensis* Richness (sOTUs)	Site (pH)	10.409	4	**0.03407**
Dataset B – *P. mascareniensis* Faith's phylogenetic diversity (PD)	Site (pH)	11.226	4	**0.02373**
Dataset B – *M. betsileanus* Richness (sOTUs)	Site (pH)	0.82648	2	0.6615
Dataset B – *M. betsileanus* Faith's phylogenetic diversity (PD)	Site (pH)	0.65833	2	0.7195
Alpha Diversity Water bacteria	**Factor**	**Glm model Type II Wald X^2**	**Degrees of freedom**	**Pr(X^2)**
Dataset Water Richness (sOTUs)	Habitat	2.3188	2	0.3137
Dataset Water Richness (sOTUs)	Habitat	0.78618	2	0.675

Table S2 – Table with values from pos-hoc multiple comparison Dunn test from the Dataset B. Statistically significant P-values are **in bold** and underlined.

Comparison	Z	P. unadj	P. adj
A - H	-0.9724213	0.33084098	1.0000000
A – R	-1.5350230	0.12477818	1.0000000
H – R	-0.3617725	0.71752204	1.0000000
A - T	-0.2378205	0.81202034	1.0000000
H - T	0.7235450	0.46934511	1.0000000
R – T	1.2134219	0.22496857	1.0000000
A – Y	1.4621608	0.14369716	1.0000000
H - Y	2.1788884	0.02933996	0.2933996
R - Y	2.9484459	0.00319376	**0.0319376**
T – Y	1.5917990	0.11142987	1.0000000

Table S3 – Table showing beta diversity explanatory factors assessed using Permutational multivariate analysis of variance (PERMANOVA, function adonis, 1000 permutations) on unweighted Unifrac distance matrix (Lozupone & Knight, 2005) to determine if Beta diversity. Significant p-values are highlighted in **bold** and underlined.

Beta Diversity (UniFrac Unweighted)	**Factors**	**Degrees of freedom**	**SumsOfSqs**	**MeanSqs**	***F* statistics**	**R squared**	**Pr (>F)**
Dataset A Skin bacteria	Species	2	0.6183	0.30914	1.08971	0.02910	0.2937
	Habitat	2	0.5697	0.28485	1.00410	0.02681	0.4346
	Species*Habitat	4	1.0532	0.26330	0.92811	0.04957	0.6404
	Residuals	67	19.0073	0.28369		0.89453	
	Total	75	21.2485			1.00000	
Dataset B *P. mascareniensis*	Site	5	2.4024	0.48048	1.7735	0.35658	**0.001998**
	Residuals	16	4.3348	0.27093		0.64342	
	Total	21	6.7372			1.00000	
Dataset B *M. betsileanus*	Site	2	0.7271	0.36353	1.1734	0.17583	0.1708
	Residuals	11	3.4079	0.30981		0.82417	
	Total	13	4.1349			1.00000	
Dataset Water bacteria	Habitat	2	0.50882	0.25441	1.256	0.26409	0.1269
	Residuals	7	1.41790	0.20256		0.73591	
	Total	9	1.92672			1.00000	

Appendix B - Supplementary material of Chapter III

Characterization of the microbiome of the invasive Asian toad in Madagascar across the expansion range and comparison with a native co-occurring species

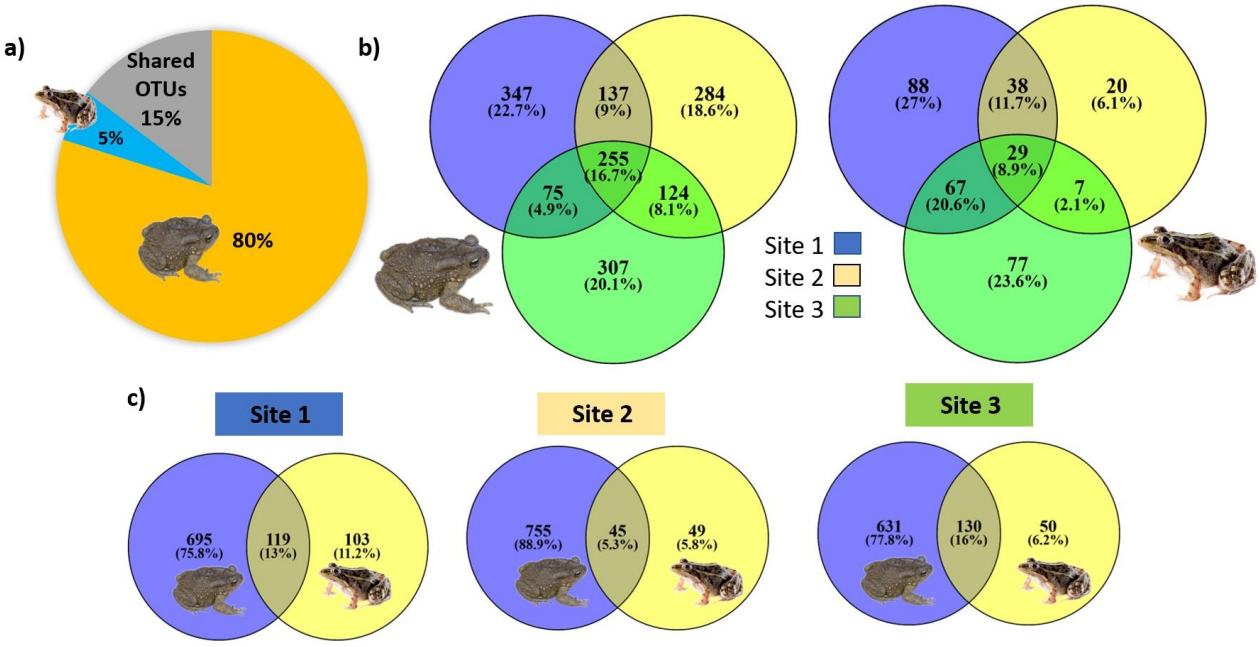


Figure S1 Skin bacterial diversity of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis*: a) Total shared and unique OTUs between the two species; b) Total shared and unique OTUs within each species across sites; c) Total shared and unique OTUs between species in each site.

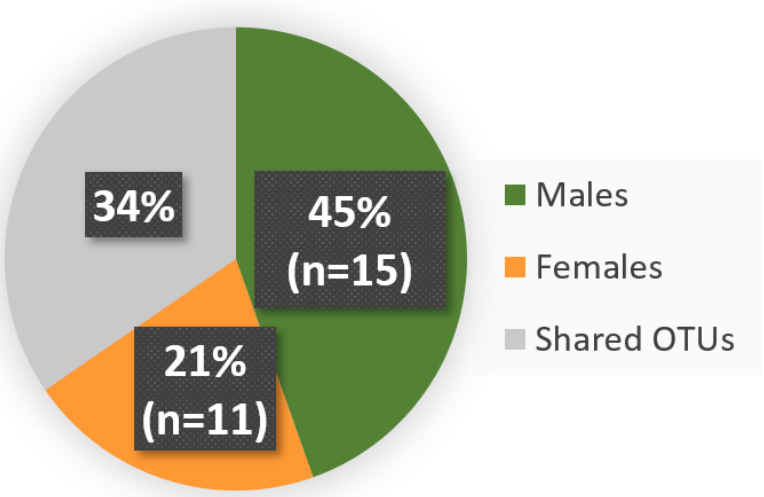


Figure S2 Total of shared and unique OTUs in males and females of *Duttaphrynus melanostictus* (individuals from the three sites were grouped).

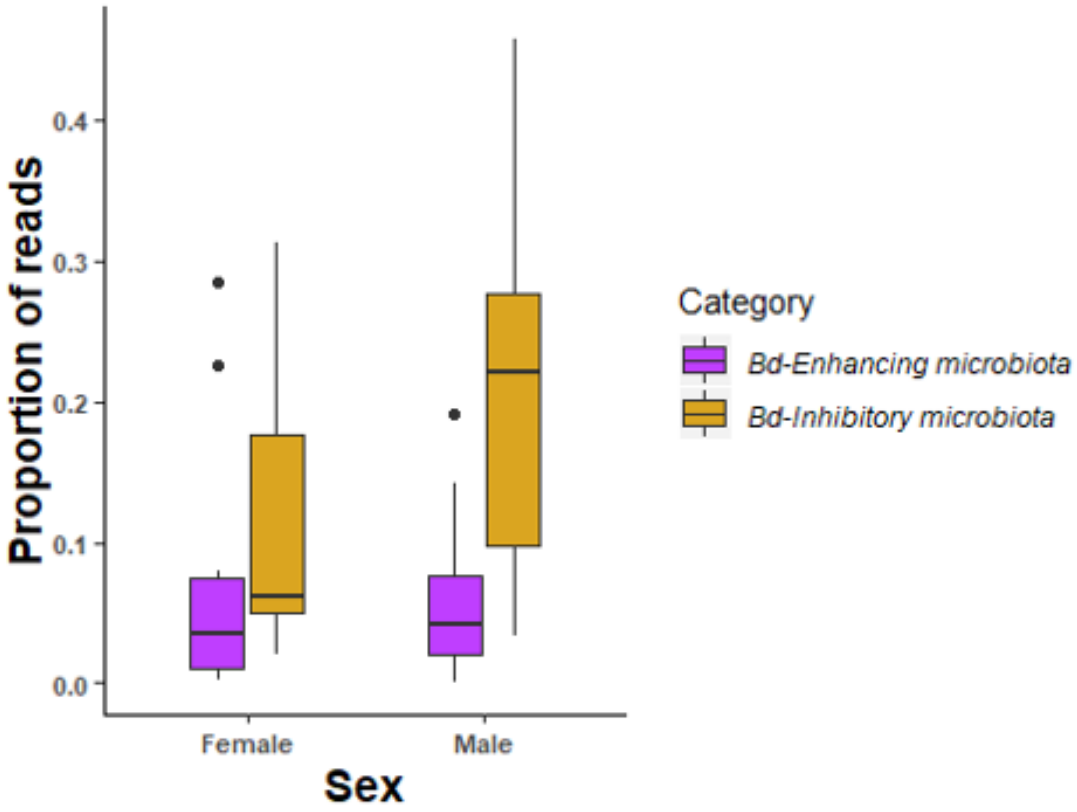
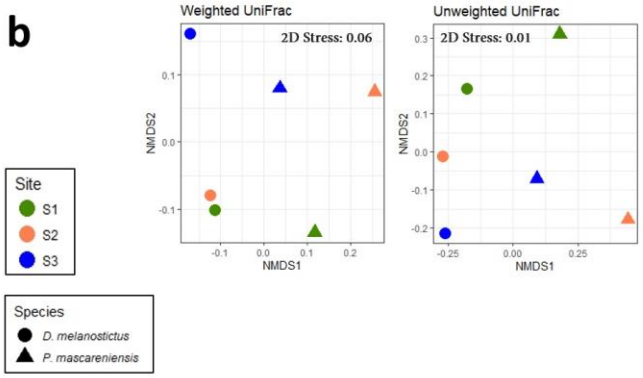


Figure S3 Proportions of reads of *Bd*-Inhibitory (yellow) and *Bd*-Enhancing (purple) skin OTUs from females and males of *Duttaphrynus melanostictus* compared to the Antifungal Isolates Database (Douglas C. Woodhams et al., 2015)

a

Species	Site	Number of individuals pooled	OTUs richness	Shannon diversity	Chao1 index	Faith' Phylogenetic diversity
Duttaphrynus melanostictus	1	4	252	7.258813	268.722	22.23789
	2	4	210	6.039202	267.468	21.50313
	3	4	133	4.348328	160.333	14.31751
Ptychadena mascareniensis	1	4	158	5.667243	205.04	17.85647
	2	4	63	2.304099	170.625	11.57004
	3	4	125	5.217796	150.869	16.7032



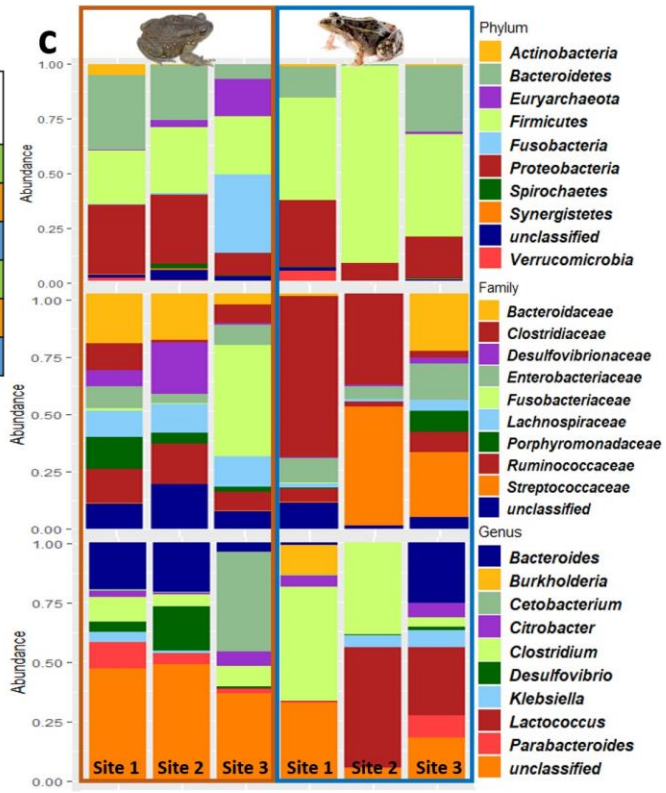


Figure S4 Gut bacterial diversity of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis*: a) Alpha Diversity metrics with no significant differences (p>0.05); b) Gut bacterial community structure of *D. melanostictus* (circles) and *P. mascareniensis* (triangles) across the 3 sites using a Non-metric multidimensional scaling (NMDS) ordination of Weighted and Unweighted Unifrac Distances; c) Composition of the gut bacterial communities including the 10 most abundant taxa from each taxonomic level (Phylum, Family and Genus) in *D. melanostictus* (left panel) and *P. mascareniensis* (right panel) across the 3 sites.

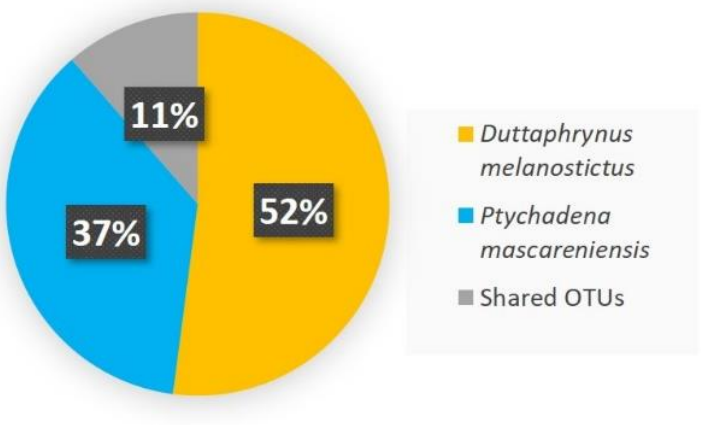


Figure S5 Total shared and unique OTUs in the gut communities of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis*. Samples from the three sampling sites were pooled together for each species.

Table S1 Total number of samples, sequences and OTUs available in each dataset before and after each filter.

	Initial values		After filtering out OTUs with less than 0.001% of total reads		Rarefaction Level	Values after Rarefaction		
	Total of sequences	Total of samples	Total of sequences	Total of samples	Rarefaction Level	Total of sequences	Number of OTUs (Deblur)	Total of samples
Skin swabs (Datasets A and B)	576,721	37	575,256	37	1,455/4,000	53,835/132,000	1,617/1,829	37/33
Gut tissue (Dataset C)	56,941	6	56,941	6	1867	11,202	701	6

Table S2 Total of samples available in datasets A, B and C after pre-processing filtering steps with QIIME. Number of samples for dataset A include total of samples with two rarefaction levels separated by "/". The pooled sample of *D. melanostictus* from each site included 2 guts from male individuals and 2 guts from female individuals; the pooled sample of *P. mascareniensis* individuals from each site included 4 guts of juveniles (sex undetermined))

DATASET A (Rarefaction: 1,455/4,000)			**DATASET B – *Only D. melanostictus* (Rarefaction: 1,455)**			**DATASET C (Rarefaction: 1,867)**		
Skin bacterial communities			**Skin bacterial communities**			**Gut bacterial communities**		
Site	*Duttaphrynus melanostictus*	*Ptychadena mascareniensis*	Site	Males	Females	Site	*Duttaphrynus melanostictus*	*P.mascareniensis*
S1	9/9	4/3	S1	3	6	S1	Pool of 4 individuals	Pool of 4 individuals
S2	10/9	3/2	S2	6	4	S2	Pool of 4 individuals	Pool of 4 individuals
S3	7/6	4/4	S3	6	1 (a)	S3	Pool of 4 individuals	Pool of 4 individuals
Total of samples	26/24	11/9	Total of samples	15	11	Total of samples	3 pools	3 pools

Notes: (a) At site 3, only 1 female was collected, so this was excluded from the statistical analysis.

Table S3 Predicted abundance of KEGG ortholog groups (Level 2 KOs) from skin bacterial communities of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis*. Groups that present significant difference (Kruskal-Wallis test) in the abundance levels between host species are colored. In yellow are the groups that were more abundant in *D. melanostictus* and in blue the groups more abundant in *P. mascareniensis*.

KEGG pathways (Level 2)	*Duttaphrynus melanostictus*	*Ptychadena mascareniensis*	% Difference (*D.melanostictus*/*P.mascareniensis*)	Kruskal-Wallis	p-value
Amino Acid Metabolism	122,871 ± 7,309	118,494 ± 5,958	3.694	3.714	0.054
Biosynthesis of Other Secondary Metabolites	10,345 ± 781	9,210 ± 1,406	12.323	5.106	0.024
Cancers	1,987 ± 319	1,843 ± 224	7.816	1.637	0.201
Carbohydrate Metabolism	120,127 ± 3,883	115,492 ± 7,917	4.013	2.439	0.118
Cardiovascular Diseases	197 ± 141	166 ± 78	19.012	0.000	1.000
Cell Growth and Death	6,713 ± 1,186	5,285 ± 791	27.013	13.851	0.000
Cell Motility	41,219 ± 10,280	41,297 ± 6,407	-0.190	0.134	0.715
Cellular Processes and Signaling	49,935 ± 5,082	57,201 ± 7,529	-12.703	7.067	0.008
Circulatory System	531 ± 154	451 ± 209	17.697	1.202	0.273
Digestive System	563 ± 181	500 ± 113	12.518	0.159	0.690
Endocrine System	4,305 ± 728	3,157 ± 819	36.352	12.175	0.000
Energy Metabolism	62,872 ± 2,430	59,216 ± 2,696	6.173	11.488	0.001
Environmental Adaptation	1,857 ± 402	1,512 ± 256	22.788	6.046	0.014
Enzyme Families	21,559 ± 1,071	23,234 ± 1,004	-7.210	14.478	0.000
Excretory System	373 ± 123	339 ± 81	10.079	0.961	0.327
Folding, Sorting and Degradation	28,094 ± 3,007	27,190 ± 1,120	3.326	0.283	0.595
Genetic Information Processing	28,305 ± 2,338	27,950 ± 1,783	1.271	0.159	0.690
Glycan Biosynthesis and Metabolism	24,857 ± 3,384	24,675 ± 2,783	0.738	0.054	0.816
Immune System	683 ± 116	694 ± 106	-1.642	0.216	0.642
Immune System Diseases	624 ± 106	603 ± 66	3.381	1.637	0.201
Infectious Diseases	5,400 ± 692	6,299 ± 1,031	-14.276	7.067	0.008
Lipid Metabolism	44,600 ± 4,336	40,721 ± 2,386	9.524	8.746	0.003
Membrane Transport	150,688 ± 19,303	175,935 ± 16,617	-14.351	10.389	0.001

Metabolic Diseases	844 ± 91	851 ± 69	-0.793	0.033	0.855
Metabolism	33,727 ± 2,544	33,087 ± 3,337	1.934	0.134	0.715
Metabolism of Cofactors and Vitamins	49,309 ± 2,384	46,805 ± 1,219	5.350	8.551	0.003
Metabolism of Other Amino Acids	23,486 ± 1,814	22,916 ± 1,172	2.491	1.276	0.259
Metabolism of Terpenoids and Polyketides	24,216 ± 2,527	21,535 ± 1,374	12.449	10.389	0.001
Nervous System	884 ± 185	1,159 ± 411	-23.727	5.106	0.024
Neurodegenerative Diseases	4,001 ± 719	3,812 ± 770	4.942	0.040	0.842
Nucleotide Metabolism	37,513 ± 1,925	36,121 ± 2,759	3.855	1.594	0.207
Poorly Characterized	65,318 ± 3,873	67,917 ± 3,919	-3.827	3.102	0.078
Replication and Repair	80,284 ± 4,807	79,728 ± 5,180	0.697	0.110	0.740
Signal Transduction	28,736 ± 2,430	31,462 ± 4,108	-8.666	4.665	0.031
Signaling Molecules and Interaction	2,084 ± 304	2,259 ± 521	-7.735	0.487	0.485
Transcription	29,913 ± 1,699	31,744 ± 1,774	-5.767	6.718	0.010
Translation	49,828 ± 5,245	45,541 ± 3,983	9.413	4.109	0.043
Transport and Catabolism	4,053 ± 532	3,408 ± 639	18.900	5.884	0.015
Xenobiotics Biodegradation and Metabolism	43,846 ± 9,770	36,934 ± 3,530	18.712	4.244	0.039

Table S4 Predicted abundance of KEGG ortholog groups (Level 2 KOs) from skin bacterial communities of males and females of *Duttaphrynus melanostictus*. Groups that show significant higher abundance levels (Kruskal-Wallis test) in males (green) and females (orange) are highlighted.

KEGG pathways (Level 2)	Males	Females	% Difference (Males/Females)	Kruskal-Wallis	*p-value*
Amino Acid Metabolism	125,184 ± 4,161	121,131 ± 10,025	3.346	2.344	0.126
Biosynthesis of Other Secondary Metabolites	10,371 ± 732	10,471 ± 871	-0.951	0.356	0.551
Cancers	2,102 ± 354	1,877 ± 254	12.008	4.639	0.031
Carbohydrate Metabolism	121,273 ± 3,285	120,009 ± 4,485	1.054	2.506	0.113
Cardiovascular Diseases	242 ± 143	140 ± 121	73.299	6.600	0.010
Cell Growth and Death	7,005 ± 1,266	6,397 ± 1,039	9.514	1.617	0.204
Cell Motility	38,441 ± 5,703	45,490 ± 13,819	-15.495	2.845	0.092
Cellular Processes and Signaling	48,929 ± 4,809	51,919 ± 5,176	-5.759	1.752	0.186
Circulatory System	514 ± 69	573 ± 226	-10.343	2.673	0.102
Digestive System	501 ± 106	657 ± 228	-23.663	2.346	0.126
Endocrine System	4,402 ± 569	4,249 ± 910	3.589	1.245	0.264
Energy Metabolism	63,993 ± 1,613	62,047 ± 3,042	3.135	2.188	0.139
Environmental Adaptation	1,735 ± 320	2,042 ± 459	-15.044	2.188	0.139
Enzyme Families	21,466 ± 1,186	21,873 ± 931	-1.859	0.297	0.586
Excretory System	394 ± 56	343 ± 180	14.894	2.344	0.126
Folding, Sorting and Degradation	26,938 ± 2,089	30,001 ± 3,261	-10.209	5.576	0.018
Genetic Information Processing	27,673 ± 1,559	29,510 ± 2,834	-6.224	3.589	0.058
Glycan Biosynthesis and Metabolism	24,040 ± 3,100	26,300 ± 3,468	-8.595	2.506	0.113
Immune System	651 ± 116	725 ± 117	-10.165	3.302	0.069
Immune System Diseases	589 ± 112	662 ± 70	-11.064	4.531	0.033
Infectious Diseases	5,272 ± 454	5,642 ± 945	-6.549	1.245	0.264
Lipid Metabolism	45,597 ± 2,412	43,876 ± 6,101	3.923	3.023	0.082
Membrane Transport	159,364 ± 16,104	140,525 ± 18,872	13.406	6.077	0.014
Metabolic Diseases	854 ± 94	858 ± 77	-0.399	0.195	0.659
Metabolism	32,988 ± 1,552	35,060 ± 3,206	-5.912	1.488	0.223

Metabolism of Cofactors and Vitamins	48,658 ± 1,733	50,750 ± 2,711	-4.121	4.418	0.036
Metabolism of Other Amino Acids	24,003 ± 1,399	23,005 ± 2,165	4.337	2.188	0.139
Metabolism of Terpenoids and Polyketides	24,779 ± 1,678	23,747 ± 3,403	4.347	2.344	0.126
Nervous System	933 ± 143	821 ± 233	13.655	2.037	0.154
Neurodegenerative Diseases	4,140 ± 585	3,813 ± 848	8.562	2.506	0.113
Nucleotide Metabolism	37,180 ± 1,781	38,351 ± 1,970	-3.053	2.673	0.102
Poorly Characterized	63,968 ± 2,873	67,882 ± 4,133	-5.766	5.097	0.024
Replication and Repair	79,284 ± 5,118	82,584 ± 3,716	-3.996	3.395	0.065
Signal Transduction	28,168 ± 1,478	29,813 ± 3,190	-5.520	3.395	0.065
Signaling Molecules and Interaction	2,243 ± 198	1,911 ± 327	17.354	6.077	0.014
Transcription	30,023 ± 1,841	30,173 ± 1,578	-0.499	0.243	0.622
Translation	48,067 ± 4,013	52,867 ± 5,811	-9.078	3.589	0.058
Transport and Catabolism	4,270 ± 374	3,823 ± 614	11.686	3.993	0.046
Xenobiotics Biodegradation and Metabolism	46,507 ± 8,030	40,828 ± 11,401	13.910	2.344	0.126

Table S5 Predicted abundance of KEGG ortholog groups (Level 2 KOs) from gut bacterial communities of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis* that present significant difference (Kruskal-Wallis test) in the abundance levels between host species. Colored in yellow are the groups that were more abundant in *D. melanostictus* and in blue groups more abundant in *P. mascareniensis*.

KEGG Pathways (Level 2)	*Duttaphrynus melanostictus*	*Ptychadena mascareniensis*	*% differences D.melanostictus/ P.mascareniensis*	Kruskal-Wallis	*p-value*
Amino Acid Metabolism	37,788 ± 1,328	36,554 ± 1,866	3.377	1.190	0.275
Biosynthesis of Other Secondary Metabolites	3,643 ± 208	2,936 ± 223	24.052	3.857	0.050
Cancers	472 ± 120	417 ± 52	13.168	0.441	0.507
Carbohydrate Metabolism	43,505 ± 2,604	41,519 ± 865	4.783	1.190	0.275
Cardiovascular Diseases	13 ± 20	7 ± 5	90.476	0.048	0.827
Cell Growth and Death	1,749 ± 99	1,672 ± 212	4.603	0.048	0.827
Cell Motility	9,141 ± 1,370	9,081 ± 1,463	0.661	0.048	0.827
Cellular Processes and Signaling	17,545 ± 1,332	17,509 ± 1,404	0.209	0.048	0.827
Circulatory System	44 ± 48	40 ± 50	10.833	0.048	0.827
Digestive System	135 ± 66	134 ± 61	0.496	0.048	0.827
Endocrine System	1,206 ± 45	881 ± 221	36.966	3.857	0.050
Energy Metabolism	22,065 ± 1,332	19,789 ± 987	11.499	3.857	0.050
Environmental Adaptation	576 ± 71	541 ± 63	6.404	0.429	0.513
Enzyme Families	8,042 ± 219	8,337 ± 282	-3.542	1.190	0.275
Excretory System	135 ± 7	121 ± 28	11.813	0.429	0.513
Folding, Sorting and Degradation	9,199 ± 142	8,694 ± 351	5.800	3.857	0.050
Genetic Information Processing	9,928 ± 742	11,126 ± 1,438	-10.767	1.190	0.275
Glycan Biosynthesis and Metabolism	9,344 ± 600	7,984 ± 1,876	17.024	0.429	0.513
Immune System	298 ± 39	283 ± 5	5.176	0.441	0.507
Immune System Diseases	205 ± 34	253 ± 49	-18.709	2.333	0.127
Infectious Diseases	1,679 ± 142	1,816 ± 378	-7.543	0.429	0.513
Lipid Metabolism	12,546 ± 730	12,152 ± 1,170	3.237	0.429	0.513
Membrane Transport	51,135 ± 896	55,072 ± 2,465	-7.148	3.857	0.050
Metabolic Diseases	353 ± 22	346 ± 63	2.019	0.048	0.827
Metabolism	11,103 ± 40	12,156 ± 962	-8.660	1.190	0.275
Metabolism of Cofactors and Vitamins	16,478 ± 873	15,974 ± 621	3.155	0.429	0.513
Metabolism of Other Amino Acids	6,706 ± 463	6,698 ± 285	0.129	0.048	0.827
Metabolism of Terpenoids and Polyketides	6,667 ± 465	6,599 ± 337	1.020	0.048	0.827
Nervous System	354 ± 36	313 ± 34	13.071	2.333	0.127

Neurodegenerative Diseases	639 ± 328	699 ± 147	-8.671	0.048	0.827
Nucleotide Metabolism	14,157 ± 1,106	14,572 ± 946	-2.843	1.190	0.275
Poorly Characterized	20,800 ± 386	20,894 ± 1,008	-0.448	0.048	0.827
Replication and Repair	29,801 ± 876	31,553 ± 1,740	-5.553	1.190	0.275
Signal Transduction	8,432 ± 429	7,666 ± 820	9.991	1.190	0.275
Signaling Molecules and Interaction	848 ± 127	892 ± 114	-4.966	0.429	0.513
Transcription	10,431 ± 901	11,533 ± 406	-9.560	2.333	0.127
Translation	18,156 ± 1,097	18,875 ± 1,377	-3.813	0.429	0.513
Transport and Catabolism	1,184 ± 295	910 ± 249	30.135	1.190	0.275
Xenobiotics Biodegradation and Metabolism	9,731 ± 1,551	9,633 ± 1,778	1.010	0.429	0.513

Table S6 Comparison of average values of bacterial richness (number of OTUs) obtained in this this study with two previous recent studies on Malagasy terrestrial and aquatic amphibians

	Number of OTUs		
	This study	Bletz *et al*., 2017a	Kueneman *et al*., 2019 (values retrieved from the plot)
Duttaphrynus melanostictus	235.9 ± 76		
Ptychadena mascareniensis	78.68 ± 53.1		
Terrestrial amphibians (Madagascar, including *P. mascareniensis*)		277.1 ± 13.9	240
Aquatic amphibians (Madagascar)		239.4 ± 14.6	125

Appendix C - Supplementary material of Chapter IV

Marked changes in skin bacterial communities match limited alterations in gut communities in metamorphosing anurans and urodele species from a small temporary pond

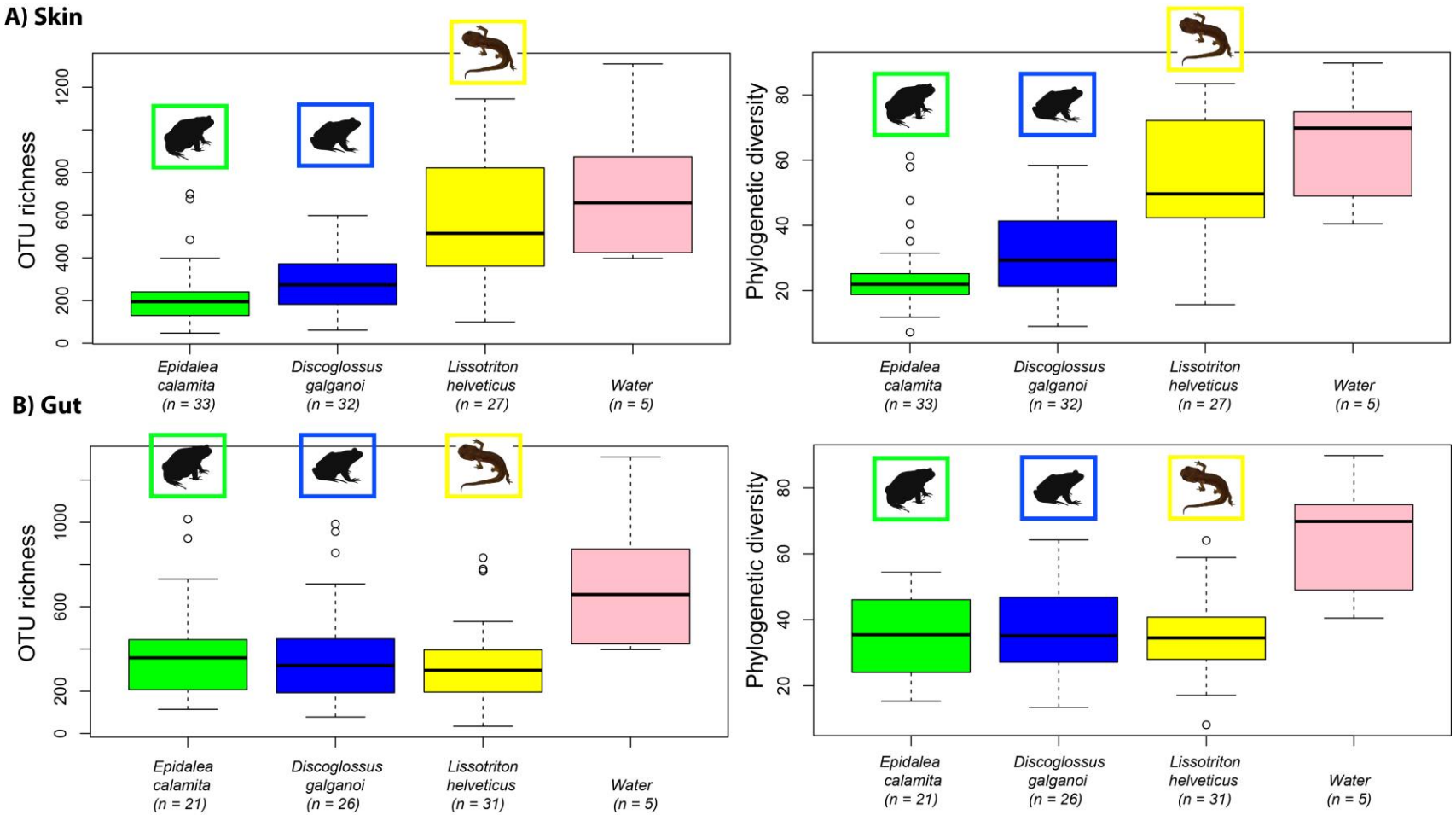


Figure S1. Alpha diversity (sOTUs richness and Phylogenetic diversity) of (A) Skin and (B) Gut communities of *Epidalea calamita* (green), *Discoglossus galganoi* (blue) and *Lissotriton helveticus* (yellow) after combining all developmental stages sampled (n indicates number of samples per species). Pink boxplots refer to water bacterial community.

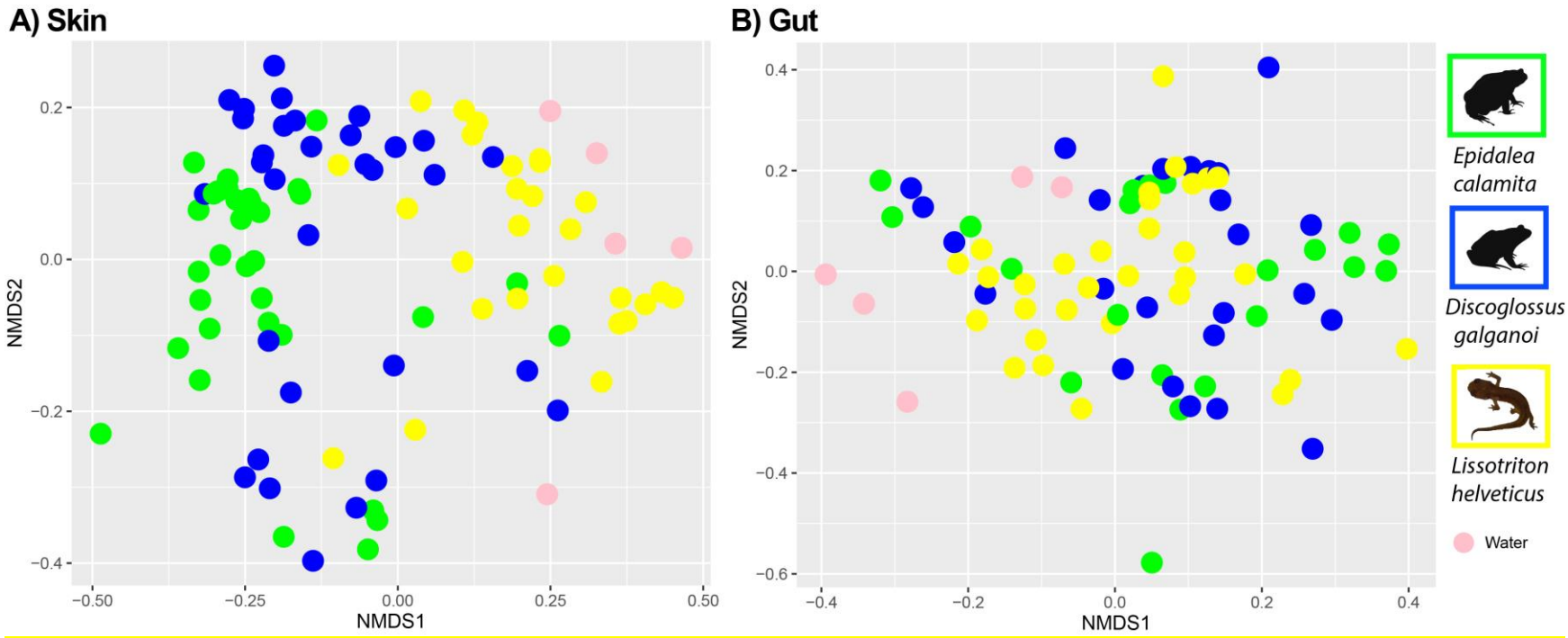


Figure S2 - Beta diversity of (A) skin and (B) gut communities of *Epidalea calamita* (green), *Discoglossus galganoi* (blue) and *Lissotriton helveticus* (yellow) after combining all developmental stages sampled. Pink circles refer to pond water bacterial community.

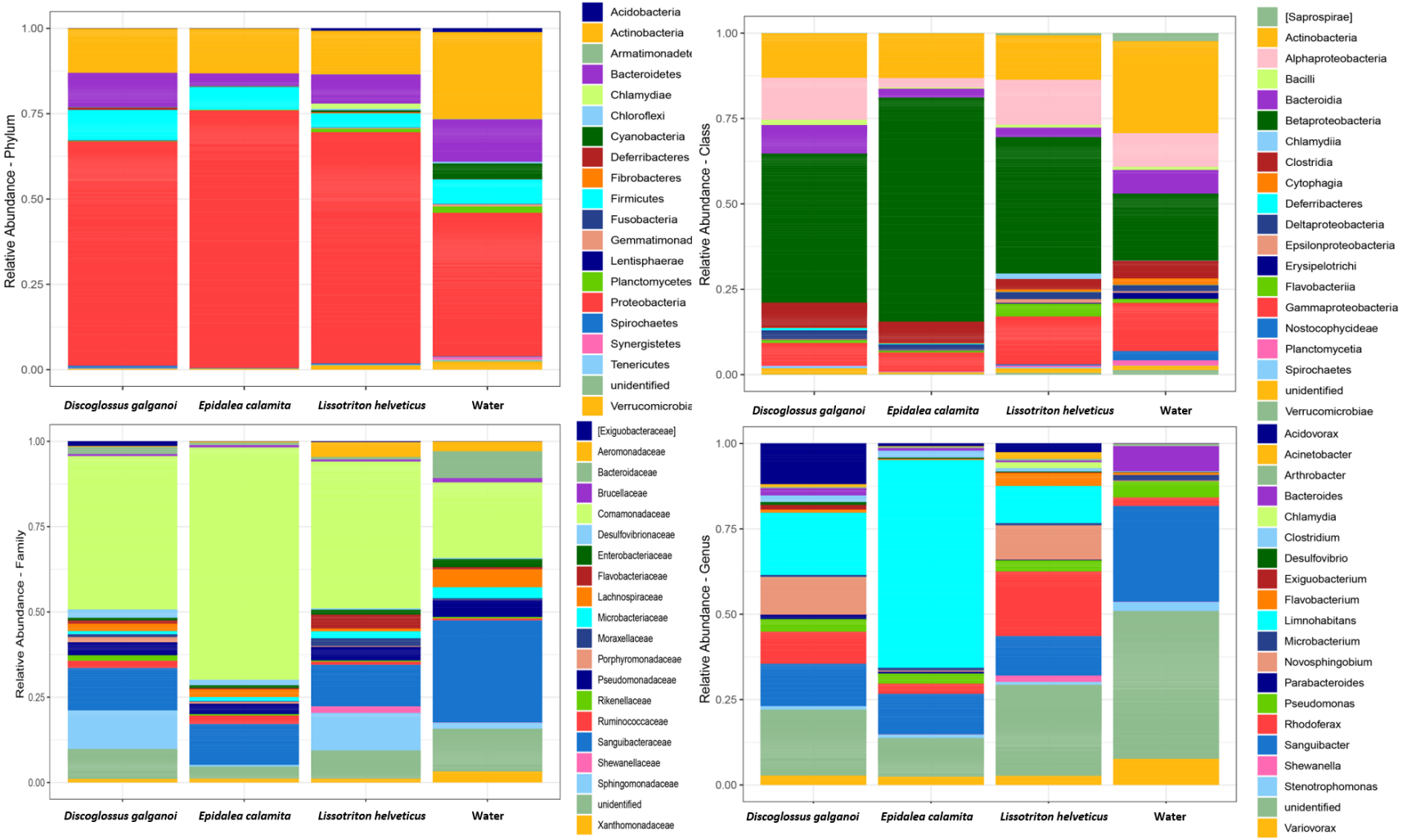


Figure S3 - Relative abundance of most prevalent 20 bacterial taxa at Phylum, Class, Family and Genus levels, from skin communities for each species and water. All developmental stages were combined within the species.

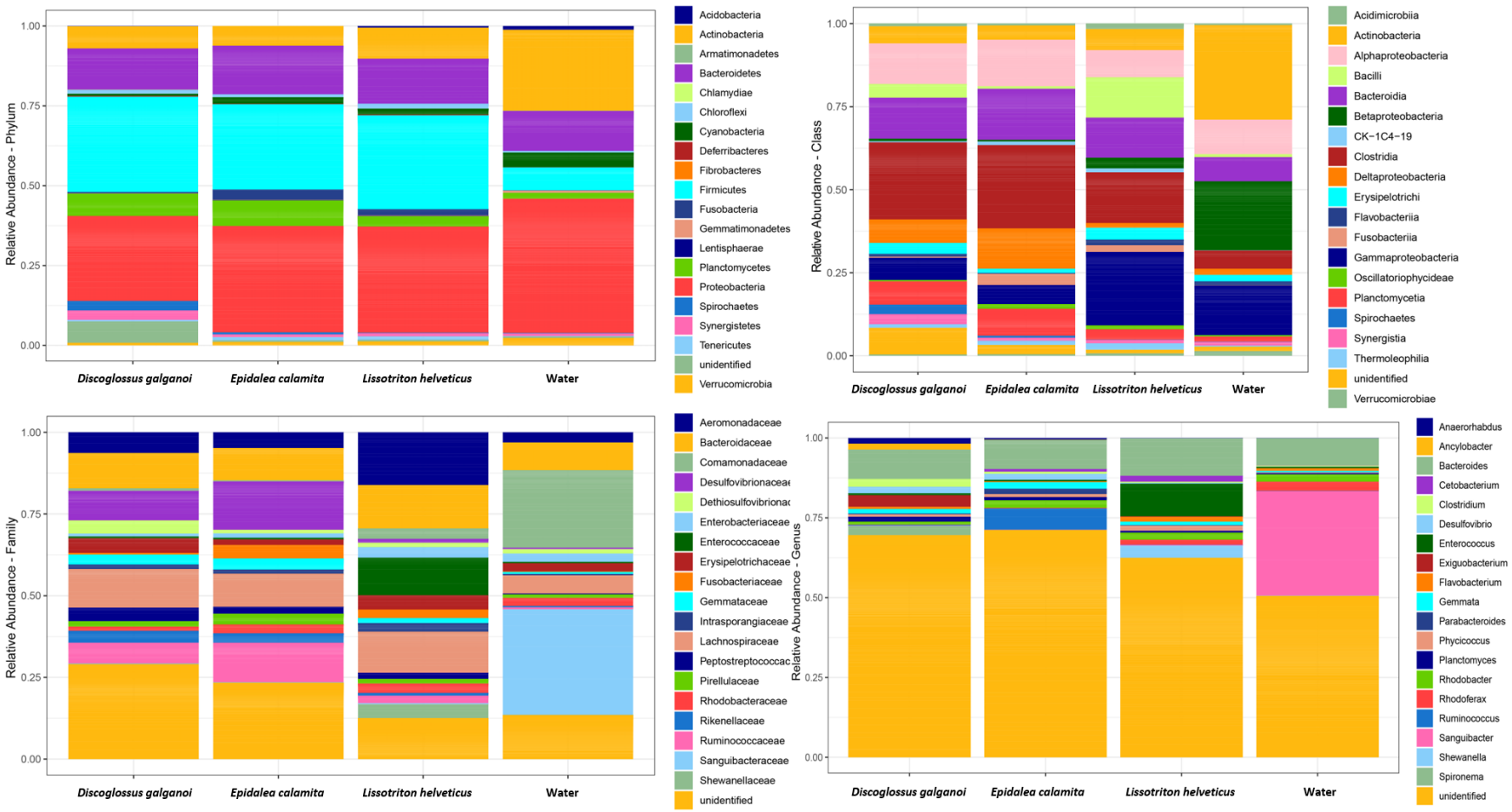


Figure S4 - Relative abundance of most prevalent 20 bacterial taxa at Phylum, Class, Family and Genus levels, from gut communities for each species and water. All developmental stages were combined within the species.

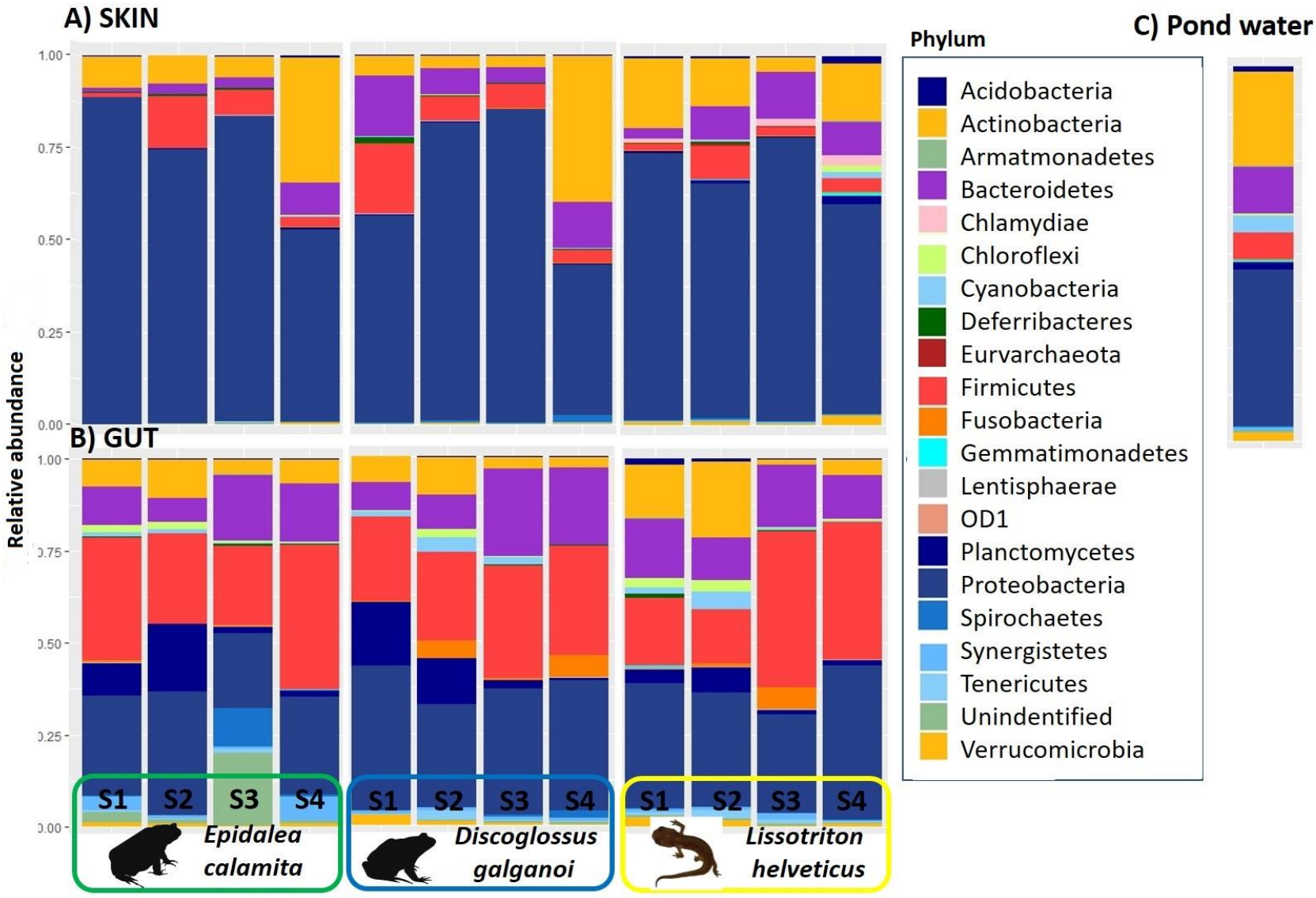


Figure S5. Relative abundance of the most abundant bacterial phylum from skin and gut communities pooled by species and life stages.

Table S1. Number of samples that passed the pre-processing filtering steps and that were used for downstream analysis

	Skin swabs					Gut tissue				
Host species	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Total**	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Total**
Epidalea calamita	9	10	7	7	33	4	7	5	5	21
Discoglossus galganoi	8	8	8	8	32	8	5	7	6	26
Lissotriton helveticus	7	6	7	7	27	6	8	9	8	31
Water swabs					5					

Table S2. Post-hoc comparisons for OTU richness and Phylogenetic diversity of skin (A) and gut (B) communities from *Epidalea calamita, Discoglossus galganoi* and *Lissotriton helveticus* and pond water. Significant differences are highlight in bold and with an asterisk.

	A - Skin bacteria		B - Gut bacteria	
Host comparison	**OTU richness**	**Phylogenetic diversity**	**OTU richness**	**Phylogenetic diversity**
E. calamita vs D. galganoi	p = 0.0483	**p = 0.0241***	p = 0.4266	p = 0.4317
E. calamita vs L. helveticus	**p = 0.0000***	**p = 0.0000***	p = 0.4082	p = 0.4907
E. calamita vs Water	**p = 0.0003***	**p = 0.0001***	p = 0.0322	**p = 0.0062***
D. galganoi vs L. helveticus	**p = 0.0002***	**p = 0.0001***	p = 0.3959	p = 0.5053
D. galganoi vs Water	**p = 0.0038***	**p = 0.0043***	p = 0.0327	**p = 0.0068***
L. helveticus vs Water	p = 0.2148	p = 0.2611	p = 0.0307	**p = 0.0088***

Table S3. Pairwise comparisons of skin and gut bacterial communities within each host species and across developmental stages using Dunn test with false discovery rate corrections. Significant differences are highlight in **bold** and marked with asterisk.

	Stages	**S1 vs S2**	**S1 vs S3**	**S1 vs S4**	**S2 vs S3**	**S2 vs S4**	**S3 vs S4**
	Host	OTU/PD	OTU/PD	OTU/PD	OTU/PD	OTU/PD	OTU/PD
Skin	*E. calamita*	0.0089*/0.0431*	0.0206*/0.1041	0.0175*/0.1221	0.5159/0.4012	0.4239/0.3700	0.5080/0.4560
	D. galganoi	0.4314/0.1262	0.1319/0.0700	0.0388/0.1578	0.0367/0.0065*	0.0317/0.0184*	0.4208/0.2879
	L. helveticus	0.1136/0.0759	0.1135/0.1135	0.0086*/0.0286	0.0070*/0.0056*	0.1115/0.3214	0.0001*/0.0014*
Gut	*E. calamita*	0.6285/0.3822	0.3866/0.3992	0.4420/0.4617	0.4446/0.3689	0.6335/0.5515	0.5076/0.4446
	D. galganoi	0.3283/0.3953	0.2331/0.4504	0.3279/0.4493	0.4958/0.5996	0.4499/0.5515	0.3751/0.4446
	L. helveticus	0.3527/0.3558	0.3510/0.2235	0.2745/0.1812	0.2214/0.1667	0.1431/0.0982	0.3333/0.4925

Table S4. Summary of PERMANOVA analysis with 9999 permutations of Unweighted UniFrac distances of host and developmental stages. Significant p-values are highlighted in **bold**.

A – Skin						
Variables	**Degrees of freedom**	**Sum of Squares**	**Mean Squares**	**F model**	**R2**	**Pr (>F)**
Host	3	4.803	1.60097	6.3327	0.14398	1e-04
Host: Life stage	9	7.320	0.81330	3.2171	0.21943	1e-04
Residuals	84	21.236	0.25281		0.63660	
Total	96	33.359			1.00000	
B - Gut						
Variables	**Degrees of freedom**	**Sum of Squares**	**Mean Squares**	**F model**	**R2**	**Pr (>F)**
Host	3	2.424	0.80796	2.1665	0.07296	1e-04
Host: Life stage	9	4.693	0.52139	1.3980	0.14125	1e-04
Residuals	70	26.106	0.37294		0.78580	
Total	82	33.222			1.000	

Table S5. Pairwise comparisons of A) skin and B) Gut bacterial communities among amphibian species using permutation analysis with 9999 permutations on a unweight Unifrac distance matrix and comparison with water samples. Significant p-values are highlighted in **bold**.

A - Skin			
	Discoglossus galganoi	***Epidalea calamita***	***Lissotriton helveticus***
Epidalea calamita	0.00015	-	-
Lissotriton helveticus	0.00015	0.00015	-
Water	0.00024	0.00015	0.02360
B - Gut			
	Discoglossus galganoi	***Epidalea calamita***	***Lissotriton helveticus***
Epidalea calamita	0.0065	-	-
Lissotriton helveticus	0.0100	0.0003	-
Water	0.0003	0.0012	0.0004
P value adjustment methods: fdr			

Table S6. Pairwise comparisons of skin bacterial communities across amphibian species and developmental stages using PERMANOVA with 9999 permutations on a unweight Unifrac distance matrix and comparison with water samples. Significant p-values are highlighted in **bold** and underlined.

		Discoglossus galganoi				***Epidalea calamita***				***Lissotriton helveticus***			
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 1	Stage 2	Stage 3	Stage 4	Stage 1	Stage 2	Stage 3	Stage 4
Discoglossus galganoi	Stage 1	-	-	-	-	-	-	-	-	-	-	-	-
	Stage 2	0.0156	-	-	-	-	-	-	-	-	-	-	-
	Stage 3	0.078	0.9126	-	-	-	-	-	-	-	-	-	-
	Stage 4	0.0078	0.0234	1	-	-	-	-	-	-	-	-	-
Epidalea calamita	Stage 1	0.0156	0.0156	0.0078	0.0078	-	-	-	-	-	-	-	-
	Stage 2	0.0078	0.0078	0.0078	0.0078	0.0156	-	-	-	-	-	-	-
	Stage 3	0.0234	0.0078	0.7254	0.0078	0.0546	0.6786	-	-	-	-	-	-
	Stage 4	0.0156	0.0234	0.2574	1	0.0156	0.0156	0.039	-	-	-	-	-
Lissotriton helveticus	Stage 1	0.0312	0.039	0.078	0.07	0.0156	0.0156	0.07	0.195	-	-	-	-
	Stage 2	0.0234	0.6786	0.195	0.0156	0.0312	0.0156	0.0468	0.0312	0.07	-	-	-
	Stage 3	0.0078	0.0234	0.0702	0.0078	0.0234	0.0078	0.0234	0.078	0.078	0.0858	-	-
	Stage 4	0.0468	0.039	0.0234	0.039	0.0234	0.0234	0.0468	0.0468	0.078	0.0234	0.0546	-
Pond water		0.0078	0.0078	0.0078	0.0078	0.0078	0.0078	0.0078	0.0234	0.0312	0.0078	0.0078	0.0702

Table S7. Pairwise comparisons of gut bacterial communities across amphibian species and developmental stages using PERMANOVA with 9999 permutations on a unweight Unifrac distance matrix and comparison with water samples. Significant p-values are highlighted in bold and underlined.

		Discoglossus galganoi				***Epidalea calamita***				***Lissotriton helveticus***			
		Stage 1	Stage 2	Stage 3	Stage 4	Stage 1	Stage 2	Stage 3	Stage 4	Stage 1	Stage 2	Stage 3	Stage 4
Discoglossus galganoi	Stage1	-	-	-	-	-	-	-	-	-	-	-	-
	Stage 2	0.738	-	-	-	-	-	-	-	-	-	-	
	Stage 3	0.021	0.0186	-	-	-	-	-	-	-	-	-	-
	Stage 4	0.283	0.0593	0.1466	-	-	-	-	-	-	-	-	-
Epidalea calamita	Stage 1	0.459	0.4525	0.0191	0.1044	-	-	-	-	-	-	-	-
	Stage 2	0.578	0.5359	0.0206	0.1214	0.3952	-	-	-	-	-	-	-
	Stage 3	0.072	0.0593	0.0186	0.098	0.1068	0.0941	-	-	-	-	-	-
	Stage 4	0.012	0.0206	0.0117	0.012	0.0206	0.0117	0.2466	-	-	-	-	-
Lissotriton helveticus	Stage 1	0.099	0.0941	0.2088	0.2426	0.0768	0.182	0.0339	0.0117	-	-	-	-
	Stage 2	0.094	0.0883	0.0186	0.04	0.0206	0.3097	0.0206	0.0117	0.578	-	-	-
	Stage 3	0.18	0.1169	0.0186	0.2088	0.0893	0.1169	0.0206	0.0117	0.151	0.0206	-	-
	Stage 4	0.051	0.04	0.0263	0.1491	0.0286	0.1004	0.0171	0.0117	0.473	0.0586	0.406	-
Pond water		0.012	0.0206	0.0117	0.0117	0.0206	0.0174	0.0206	0.0206	0.012	0.0117	0.012	0.0117

Table S8. Pairwise comparisons of skin and gut bacterial communities across amphibian species and the two most extreme developmental stages (stage 1 and stage 4) using PERMANOVA with 9999 permutations on a unweight Unifrac distance matrix and comparison with water samples. Significant p-values are highlighted in bold and underlined.

			Discoglossus galganoi				***Epidalea calamita***				***Lissotriton helveticus***			
			Stage 1		Stage 4		Stage 1		Stage 4		Stage 1		Stage 4	
			Gut	Skin	Gut	Skin	Gut	Skin	Gut	Skin	Gut	Skin	Gut	Skin
Discoglossus galganoi	Stage 1	Skin	0.00104	-	-	-	-	-	-	-	-	-	-	-
	Stage 4	Gut	0.2287658	0.0013	-	-	-	-	-	-	-	-	-	-
		Skin	0.0013929	0.000975	0.0014757	-	-	-	-	-	-	-	-	-
Epidalea calamita	Stage 1	Gut	0.5181	0.0034373	0.0977167	0.0041516	-	-	-	-	-	-	-	-
		Skin	0.000975	0.000975	0.00104	0.00104	0.0026	-	-	-	-	-	-	-
	Stage 4	Gut	0.0014625	0.0018571	0.0034373	0.0013929	0.0106364	0.0014757	-	-	-	-	-	-
		Skin	0.00117	0.000975	0.0014757	0.1243784	0.0040918	0.00117	0.0033429	-	-	-	-	-
Lissotriton helveticus	Stage 1	Gut	0.0844817	0.00117	0.172848	0.0014625	0.04602	0.0013	0.0027	0.0014757	-	-	-	-
		Skin	0.00117	0.000975	0.0013929	0.0022286	0.00351	0.00104	0.00195	0.00208	0.0027444	-	-	-
	Stage 4	Gut	0.0366261	0.000975	0.1243784	0.00104	0.0173206	0.000975	0.0021574	0.00104	0.3437065	0.0014625	-	-
		Skin	0.0013	0.00117	0.0013929	0.0016	0.0042095	0.000975	0.0021574	0.0014757	0.0016	0.0014625	0.00104	-
Pond water			0.0029782	0.0018571	0.0034211	0.001755	0.0101156	0.0013	0.0106364	0.0110597	0.0027444	0.00195	0.00234	0.0022286

Appendix D - Supplementary material of Chapter V

The effect of water source on tadpole microbiome from different species in a short-term experiment using 16S metabarcoding

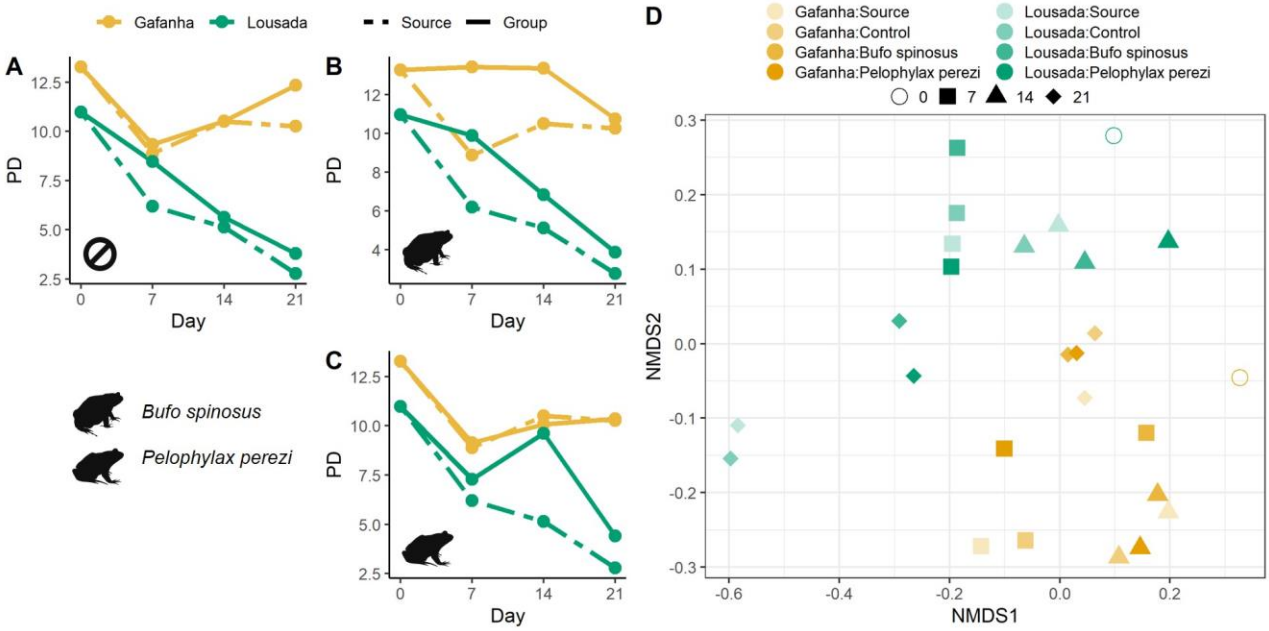


Figure S1 Alpha (A-C) and Beta (D) diversities of the bacterial communities occurring in water samples used in the experiment over time (day 0 to 21). A-C show the variation in phylogenetic diversity observed in the water from each experimental group (solid lines) – control (A), *Bufo spinosus* (B) and *Pelophylax perezi* (C), comparatively to the bacterial communities occurring in the natural waterbodies (Source; dashed line); and D present the β-diversity based on the unweighted unifrac distances among all water samples from each experimental group. Colors represent the two source waters – Gafanha (yellow) and Lousada (green). In D, the different shades distinguish the bacterial communities observed within the experimental groups associated to each source water (control, *B. spinosus*, *P. perezi*), and symbols represent the different sampling events.

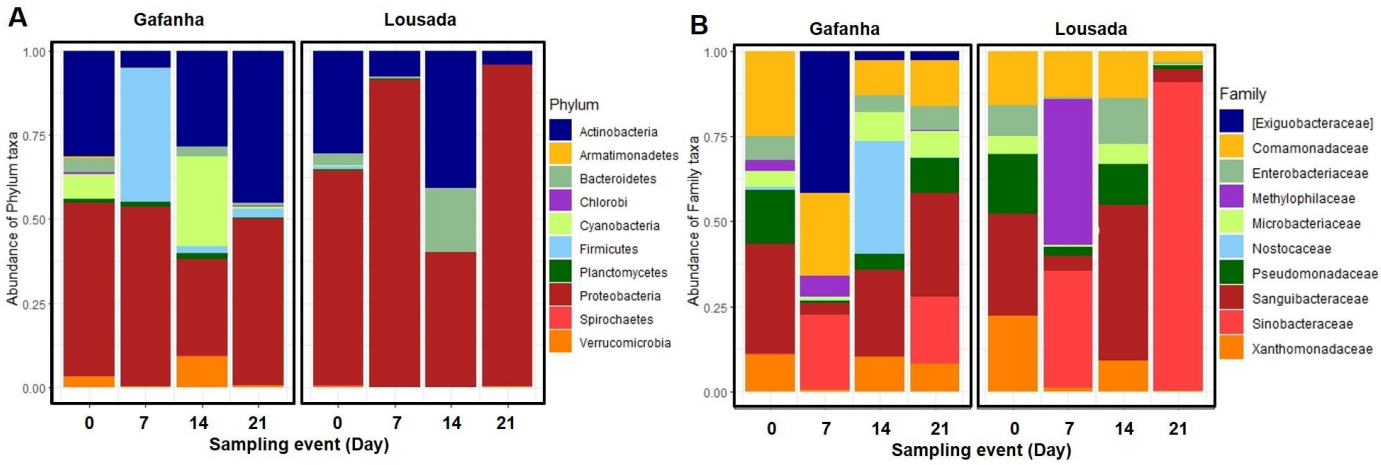


Figure S2 Abundance of the 10 most prevalent bacterial Phyla (A) and Families (B) detected in each water source that were collected in each source water (Gafanha and Lousada) across the sampling events (Days).

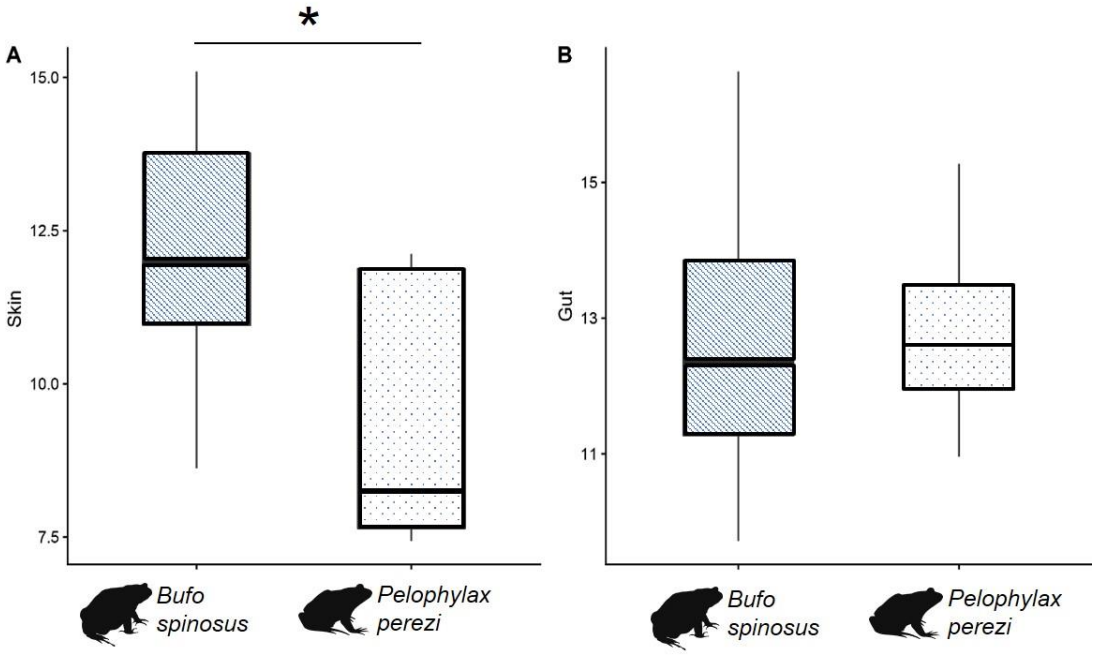


Figure S3 - Boxplots of bacterial α-diversity (using the Faith' phylogenetic diversity metric) detected in the skin (A) and gut (B) of *Bufo spinosus* and *Pelophylax perezi* tadpoles at day 0. Asterisk denotes significant differences between groups.

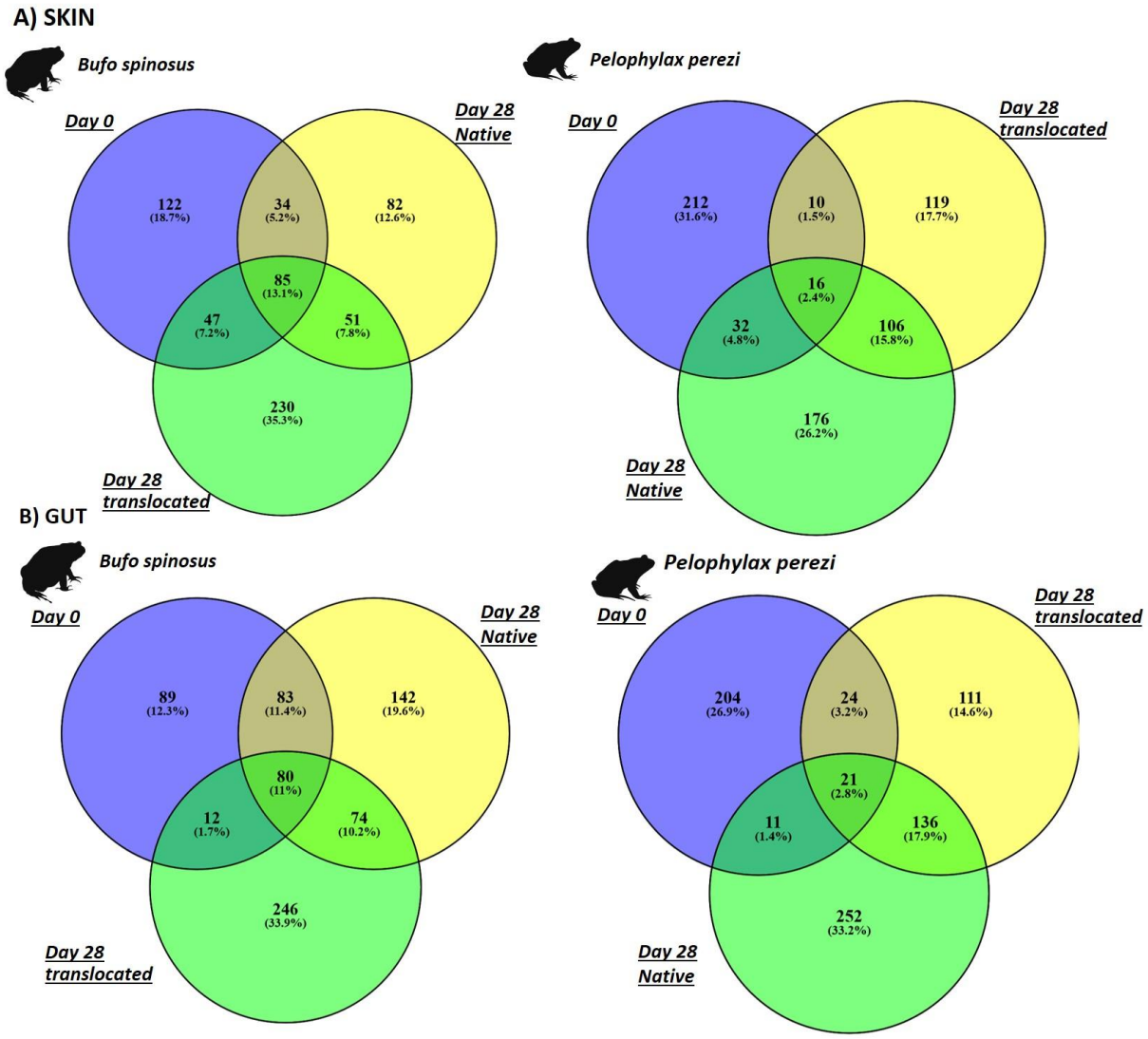


Figure S4 - Total of unique and shared bacterial OTUs on the skin (A) and gut (B) of tadpoles of each species at day 0 and day 28 exposed to the two water sources.

Table S1 Summary of GLMM statistics used to evaluate the effect of source water (native or translocated), sampling events (Days 0/7/14/21/28) and interaction of both factors on phylogenetic diversity of (A) skin bacteria of *Pelophylax perezi*; (B) skin bacteria of *Bufo spinosus*; (C) gut bacteria of *P. perezi*, and (D) gut bacteria of *B. spinosus. P.perezi* tadpoles were collected at Gafanha and *B. spinosus* tadpoles at Lousada, which correspond to their native source waters respectively.. For each model, we provide the parameter estimates, standard errors (SE), the t value and statistical significance of terms (Pr). Significant values are highlight in bold.

SKIN				
(A)P. perezi	Estimate	SE	t value	Pr(>|t|)
(Intercept)	8.540804	1.078920	7.916	0.000149 ***
OriginTranslocated	-1.038880	1.521489	-0.683	0.518588
Day	0.165331	0.056777	2.912	**0.006670 ****
OriginTranslocated:Day	0.003387	0.078791	0.043	0.965986
(B)B. spinosus	Estimate	SE	t value	Pr(>|t|)
(Intercept)	12.47543	0.80507	15.496	<2e-16 ***
OriginTranslocated	-0.43842	1.14205	-0.384	0.70254
Day	-0.02824	0.04695	-0.601	0.54998
OriginTranslocated:Day	0.20556	0.06762	3.040	**0.00362 ****
GUT				
(C)P. perezi	Estimate	SE	t value	Pr(>|t|)
(Intercept)	13.30007	0.80315	16.560	**<2e-16 *****
OriginTranslocated	-1.07791	1.15341	-0.935	0.356
Day	0.01751	0.04190	0.418	0.679
OriginTranslocated:Day	-0.04529	0.06073	-0.746	0.461
(D)B. spinosus	Estimate	SE	t value	Pr(>|t|)
(Intercept)	13.33373	1.40983	9.458	**0.000264 *****
OriginTranslocated	-3.72775	1.95500	-1.907	0.119137
Day	0.03260	0.06528	0.499	0.621859
OriginTranslocated:Day	0.22436	0.09174	2.446	**0.021230 ***

Table S2 Summary of nonparametric permutational multivariate analysis of variance (PERMANOVA; 9,999 permutations) used to evaluate the effects of source water (Gafanha or Lousada), host species (*B. spinosus* or *P. perezi*) and sampling events (Day 0/7/14/21/28) on bacterial composition of (A) Skin; (B) Gut. *P.perezi* tadpoles were collected at Gafanha and *B. spinosus* tadpoles at Lousada, which correspond to their native source waters respectively. Significant values are highlight in bold.

(A) SKIN	**df**	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**
Group	1	2.786	2.78559	11.4097	0.08093	**1e-04*** **
Group: Origin	2	1.794	0.89712	3.6746	0.05213	**1e-04*** **
Group: Origin: Day	4	2.986	0.74645	3.0574	0.08674	**1e-04*** **
Residuals	110	26.856	0.24414		0.78021	
Total	117	34.421			1.00000	
(B) GUT	**df**	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**
Group	1	1.5686	1.56864	6.1469	0.05975	**1e-04*** **
Group: Origin	2	2.7119	1.35594	5.3134	0.10330	**1e-04*** **
Group: Origin: Day	4	2.8326	0.70815	2.7750	0.10790	**1e-04*** **
Residuals	75	19.1395	0.25519		0.72905	
Total	82	26.2526			1.00000	

*** p<0.001, ** p<0.01, * p<0.05, ^{ns} p>0.05

Table S4 Identification of core100 OTUs that were associated to each host species (*Bufo spinosus* and *Pelophylax perezi*) and shared among the three experimental groups (day0, day28-native, day28-translocated) occurring in the skin or gut.

Host species	Phylum	Class	Family	Genus/ Species
SKIN				
Bufo spinosus	Actinobacteria	Actinobacteria	Microbacteriaceae	*unidientified*
	Proteobacteria	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas fragi*
	Actinobacteria	Actinobacteria	Microbacteriaceae	*Microbacterium maritypicum*
	Proteobacteria	Gammaproteobacteria	Enterobacteriaceae	*unidientified*
	Proteobacteria	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas veronii*
	Proteobacteria	Betaproteobacteria	Comamonadaceae	*unidentified*
	Proteobacteria	Gammaproteobacteria	Xanthomonadaceae	*Stenotrophomonas*
	Proteobacteria	Betaproteobacteria	Comamonadaceae	*Variovorax paradoxus*
	Actinobacteria	Actinobacteria	Sanguibacteraceae	*Sanguibacter*
Pelophylax perezi	Proteobacteria	Betaproteobacteria	Comamonadaceae	*Variovorax paradoxus*
	Proteobacteria	Betaproteobacteria	Comamonadaceae	*Limnohabitans curvus*
GUT				
Bufo spinosus	Proteobacteria	Alphaproteobacteria	unidientified	*unidientified*
	Planctomycetes	Planctomycetia	Gemmataceae	*unidientified*
	Planctomycetes	Planctomycetia	Isosphaeraceae	*unidientified*
	Proteobacteria	Alphaproteobacteria	unidientified	*unidientified*
	Proteobacteria	Alphaproteobacteria	unidientified	*unidientified*
	Actinobacteria	Actinobacteria	Mycobacteriaceae	*Mycobacterium*
Pelophylax perezi	None			

Appendix E - Front page of other research papers published during the doctoral program

E1 – Pinho et al., 2018

genes

MDPI

Article

What Is the Giant Wall Gecko Having for Dinner? Conservation Genetics for Guiding Reserve Management in Cabo Verde

Catarina Jesus Pinho [1,2], Bárbara Santos [1,2], Vanessa A. Mata [1,2], Mariana Seguro [1], Maria M. Romeiras [3,4], Ricardo Jorge Lopes [1] and Raquel Vasconcelos [1,*]

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check for updates

Abstract: Knowledge on diet composition of a species is an important step to unveil its ecology and guide conservation actions. This is especially important for species that inhabit remote areas within biodiversity hotspots, with little information about their ecological roles. The emblematic giant wall gecko of Cabo Verde, *Tarentola gigas*, is restricted to the uninhabited Branco and Raso islets, and presents two subspecies. It is classified as Endangered, and locally Extinct on Santa Luzia Island; however, little information is known about its diet and behaviour. In this study, we identified the main plant, arthropods, and vertebrates consumed by both gecko subspecies using next generation sequencing (NGS) (metabarcoding of faecal pellets), and compared them with the species known to occur on Santa Luzia. Results showed that plants have a significant role as diet items and identified vertebrate and invertebrate taxa with higher taxonomic resolution than traditional methods. With this study, we now have data on the diet of both subspecies for evaluating the reintroduction of this threatened gecko on Santa Luzia as potentially successful, considering the generalist character of both populations. The information revealed by these ecological networks is important for the development of conservation plans by governmental authorities, and reinforces the essential and commonly neglected role of reptiles on island systems.

Keywords: Desertas Islands; conservation; diet; metabarcoding; protected areas; *Tarentola gigas*

E2 – Lopes et al., 2019

Received: 8 October 2018 | Revised: 20 February 2019 | Accepted: 1 March 2019

DOI: 10.1002/ece3.5105

ORIGINAL RESEARCH

WILEY Ecology and Evolution

Intricate trophic links between threatened vertebrates confined to a small island in the Atlantic Ocean

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Abstract

Trophic networks in small isolated islands are in a fragile balance, and their disturbance can easily contribute toward the extinction vortex of species. Here, we show, in a small Atlantic island (Raso) in the Cabo Verde Archipelago, using DNA metabarcoding, the extent of trophic dependence of the Endangered giant wall gecko *Tarentola gigas* on endemic populations of vertebrates, including one of the rarest bird species of the world, the Critically Endangered Raso lark *Alauda razae*. We found that the Raso lark (27%), Iago sparrow *Passer iagoensis* (12%), Bulwer's petrel *Bulweria bulwerii* (15%), and the Cabo Verde shearwater *Calonectris edwardsii* (10%) are the most frequent vertebrate signatures found in the feces of the giant wall gecko. This work provides the first integrative assessment of their trophic links, an important issue to be considered for the long-term conservation of these small and isolated island ecosystems.

KEYWORDS
birds, Cabo Verde, DNA metabarcoding, endemics, reptiles, trophic networks

1.

E3 – Pereira et al., 2021

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Application of a standard risk assessment scheme to a North Africa contaminated site (Sfax, Tunisia) -Tier 1

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HIGHLIGHTS

- Tier 1 of risk assessment framework may include only two lines of evidence (LoE).
- Soil salinity accounted for the lack of coherence between LoE.
- Different weights given to each LoE may overcome the confounding effect of salinity.

GRAPHICAL ABSTRACT

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ABSTRACT

Phosphorus is a critical element to agriculture, consequently global phosphate rock demand will remain rising to feed a growing world population. The beneficiation of phosphorous ore gives rise to several tons of a waste by-product [phosphogypsum (PG)] which valorisation is limited, within other reasons, by the risks posed to environment and human health. Although threatening, the accumulation in stacks is the only procedure so far practiced by several countries as a means to get rid of this industrial externality. As part of a NATO Science for Peace Project (SfP 983111) this study describes the application of an environmental risk assessment (ERA) framework, to assess the risks posed by a PG stack to the surrounding soils, in Sfax, Republic of Tunisia. The ERA followed a weight of evidence approach, supported by two lines of evidence (LoE): the chemical (ChemLoE) and the ecotoxicological (EcotoxLoE). Integrated risks point for risk values greater than 0.5 in soils collected in PG stack surrounding area. Soil salinization, has

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2.

E4 - Santos et al., 2021

Journal of Arachnology (accepted)

Diet study of geckos reveals the first records of pseudoscorpions on Desertas, Cabo Verde Islands

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Abstract:

Pseudoscorpions are small invertebrates, hard to detect and poorly studied. With circa 3700 extant species and worldwide distribution, they hold higher diversity in the tropics and subtropics. Among their predators are ground-dwelling taxa, such as arthropods, amphibians, small birds, and reptiles. Only four pseudoscorpion species are known in the Cabo Verde Archipelago, but none in the Desertas Islands. In this study, we record the first two species for the Desertas Islands. We used molecular and morphological methods to taxonomically identify the specimens retrieved from reptile faecal pellets and pitfalls. We identified the presence of Garypus cf. saxicola on Raso Islet, of Olpium pallipes on Raso and Santa Luzia Island and a putative new Olpium species on Branco Islet. This study emphasizes how non-invasive sampling combined with metabarcoding and morphological studies can be used to uncover unknown biodiversity, particularly of cryptic groups from inaccessible locations.

Keywords: Arachnida, genetics, morphology, Pseudoscorpiones, Raso, Branco and Santa Luzia Island

E5 – Other papers submitted or under revision

1. **Mors tua, vita mea: feeding frenzy in painted frog tadpoles eating fire salamander larvae in an ephemeral pond**

   (Submitted in October 2020)

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2. **Assessing stress response in lizards from agroecosystems with different management practices.**

   (Submitted in November 2020)

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