ORIGINAL ARTICLE

Encouraging news for in situ conservation: Translocation of salamander larvae has limited impacts on their skin microbiota

Léa Fieschi-Méric[1,2] | Pauline van Leeuwen[2,3] | Mathieu Denoël[1] | David Lesbarrères[2,4]

[1]Laboratory of Ecology and Conservation of Amphibians (LECA), Freshwater and OCeanic science Unit of reSearch (FOCUS), Université de Liège, Liège, Belgium

[2]Biology Department, Laurentian University, Sudbury, Ontario, Canada

[3]Conservation Genetics Laboratory, University de Liège, Liège, Belgium

[4]Environment and Climate Change Canada, Ottawa, Ontario, Canada

Correspondence
Léa Fieschi-Méric, Laboratory of Ecology and Conservation of Amphibians (LECA), Freshwater and OCeanic science Unit of reSearch (FOCUS), Université de Liège, Liège, Belgium.
Email: leafieschimeric@gmail.com

Abstract

The key role of symbiotic skin bacteria communities in amphibian resistance to emerging pathogens is well recognized, but factors leading to their dysbiosis are not fully understood. In particular, the potential effects of population translocations on the composition and diversity of hosts' skin microbiota have received little attention, although such transfers are widely carried out as a strategy for amphibian conservation. To characterize the potential reorganization of the microbiota over such a sudden environmental change, we conducted a common-garden experiment simulating reciprocal translocations of yellow-spotted salamander larvae across three lakes. We sequenced skin microbiota samples collected before and 15 days after the transfer. Using a database of antifungal isolates, we identified symbionts with known function against the pathogen *Batrachochytrium dendrobatidis*, a major driver of amphibian declines. Our results indicate an important reorganization of bacterial assemblages throughout ontogeny, with strong changes in composition, diversity and structure of the skin microbiota in both control and translocated individuals over the 15 days of monitoring. Unexpectedly, the diversity and community structure of the microbiota were not significantly affected by the translocation event, thus suggesting a strong resilience of skin bacterial communities to environmental change—at least across the time-window studied here. A few phylotypes were more abundant in the microbiota of translocated larvae, but no differences were found among pathogen-inhibiting symbionts. Taken together, our results support amphibian translocations as a promising strategy for this endangered animal class, with limited impact on their skin microbiota.

KEYWORDS
amphibian conservation, *Batrachochytrium dendrobatidis*, common-garden experiment, holobiont conservation, microbiota flexibility, population translocation

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Mathieu Denoël and David Lesbarrères share senior authorship.

1 | INTRODUCTION

Amphibians are currently the most endangered animal class on earth, with over one third of extant species threatened with extinction (Stuart et al., 2004). Among many other threats, the rapid spread of emerging diseases, facilitated by globalization, animal trade and climate change, is outstandingly preoccupying (Cardiel et al., 2008; Wake & Vredenburg, 2008). For example, the deadly "chytridiomycosis" has caused mass amphibian die-offs worldwide, with declines in over 500 amphibian species, including 90 presumed extinctions in the last 50 years (Scheele et al., 2019). This disease is caused by two fungi, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*), which disrupt the amphibian skin and impair its essential homeostatic functions, leading to the death of heavily infected individuals (Fisher & Garner, 2020; Longcore et al., 1999; Martel et al., 2013). Interestingly, both pathogens can suppress immune responses in their host (Fites et al., 2013; Rollins-Smith & Le Sage, 2021), and the resistance observed in some amphibians has been suggested to arise from bacterial symbionts present on their skin (Rebollar et al., 2020; Vredenburg et al., 2011).

The role of resident micro-organisms on the health and resistance of their host to infectious diseases is increasingly recognized (Libertucci & Young, 2019; Stecher & Hardt, 2008). In this context, several species of bacteria that produce metabolites inhibiting the growth of amphibian pathogenic fungi have been identified (Brucker et al., 2008; Lauer et al., 2008; Woodhams et al., 2018). However, these symbionts are not present in all amphibians: the composition and diversity of amphibian skin bacterial communities, referred to as "skin microbiota," varies between species, individuals and even life stages (Jiménez & Sommer, 2017; Kueneman et al., 2014; Sabino-Pinto et al., 2017). As a consequence, slight differences in microbiota structure among populations from the same species can be associated with divergent disease outcomes (Bates et al., 2018). The popularization of high-throughput sequencing of marker genes, such as the bacterial 16S rRNA gene, has recently allowed the extensive characterization of the amphibian microbiota and its natural plasticity (Shendure & Ji, 2008). Indeed, amphibian skin microbiota are dynamic, and are influenced by extrinsic factors and by the bacteria present in their environment (Harrison et al., 2019; Walke et al., 2014). However, strong environmental changes such as habitat alteration or pollution can lead to the disruption of the microbiota. These alterations, referred to as "dysbiosis," can have a negative effect on the host, as they may facilitate infection by opportunistic pathogens (Croswell et al., 2009; Jiménez & Sommer, 2017).

In view of global and rapid declines in amphibia, one of the most recommended strategies for their conservation consists of population translocation, defined by the International Union for Conservation of Nature (IUCN) as the "human-mediated movement of organisms from one site to another, where the primary objective is a conservation benefit" (IUCN/SSC, 2013). Most translocation efforts consist of removing individuals from threatened habitats ("mitigation translocation") and/or reintroducing populations in sites where they have disappeared or are declining ("population restoration";

Linhoff et al., 2021). Despite their common use for amphibian conservation, translocations have a limited success: only 20% are associated with evidence of reproduction during several years following release (Scheele et al., 2021). Many translocation failures have been attributed to chytridiomycosis (Scheele et al., 2021): the potential increased susceptibility to this disease could result from a strong dysbiosis of the skin microbiota caused by rapid habitat change, or, conversely, from a lack of adaptation of the microbiota to the new environment. Characterizing the dynamics of rearrangement of the amphibian skin microbiota following translocations could therefore be essential to increase the success of this conservation method.

A few recent studies in diverse taxa have shown the important plasticity of gut and skin microbiota in response to translocations. Bacterial communities rapidly shift in composition towards a microbiota similar to that of individuals in the environment of translocation, while remaining distinguishable, sometimes for several months (Uren Webster et al., 2020; van Leeuwen et al., 2020; Yao et al., 2019). In amphibians, scarce evidence suggests that translocations can induce changes in the structure of bacterial communities (Bletz et al., 2016; Kueneman et al., 2022; Nolan, 2020); however, their impact on fungi-inhibiting phylotypes remains to be explored.

Here, we translocated yellow-spotted salamander larvae, *Ambystoma maculatum* (Shaw, 1802), using an in situ common-garden experimental design to investigate the effects of this conservation method on the amphibian skin microbiota. Indeed, amphibian conservation-translocations are often undertaken using young life stages to reduce rearing and infrastructure costs (Linhoff et al., 2021). In particular, we examined the dynamics of reorganization of fungi-inhibiting bacterial communities using the Antifungal Isolates Database (which compiles all bacterial sequences known to inhibit *Bd*; Woodhams et al., 2015) to determine whether the translocation would cause their dysbiosis and thus infer potential consequences on translocation success. Indeed, while larval *A. maculatum* are probably resistant to *Bd*, it is unknown whether their resistance to this pathogen arises from skin secretions or from symbiotic skin bacteria (Crawshaw et al., 2022; Martel et al., 2014); in the latter scenario, potential variation in the *Bd*-inhibitory microbiota caused by the translocation could have serious implications for their survival. Since infection status is known to affect bacterial community structures, we also screened our samples for the presence of major amphibian pathogens (i.e., ranaviruses and chytrids). While probably resistant to *Bd* and *Bsal*, larval *A. maculatum* could act as reservoirs for these fungi (Crawshaw et al., 2022; Martel et al., 2014), and the fact that ranaviruses can be lethal in other *Ambystoma* suggests that yellow-spotted salamanders might also be susceptible to these pathogens (Brunner et al., 2011; Picco et al., 2007; Rojas et al., 2005).

We hypothesized that the microbiota of transferred individuals would rapidly converge towards that of control individuals in the lake of destination. Based on historical records (Brunner et al., 2021; Crawshaw et al., 2022), we also expected a low prevalence of pathogens in our lakes of interest. As a consequence of the resulting weak selection pressure for protective bacteria, we predicted that the proportion of *Bd*-inhibitory phylotypes might be low in our sampled

populations. We discuss the possible implications of microbiota restructuration on the fitness of their host and the success of population translocation as a conservation strategy.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Reciprocal transplants were conducted between three lakes in Algonquin Provincial Park, Canada: Bat Lake (BL, 45°35′10″N, 78°31′07″W, 400 m elev.), Lost Ray Lake (LL, 45°35′25″N, 78°32′13″W, 416 m elev.) and Speckled Trout Lake (SP, 45°31′59″N, 78°25′28″W, 420 m elev.). These locations were chosen in a radius of less than 6 km to reduce uncontrolled climatic variation between sites. A total of 63 mesocosms (60 × 20 cm), custom-made from nylon bags stitched spread opened inside minnow traps, were equally distributed between the three lakes. Each mesocosm was attached to a stake on the shore, so that two thirds of the volume inside the nylon bag were submerged, and a handful of substrate from the lake was added to the bottom of each bag. The mesh of the nylon (2 mm in diameter) allowed cladocerans and copepods to pass through, thus providing food for the salamander larvae (Freda, 1983; Figure 1a).

Twenty-one spotted salamander larvae at developmental stage 45 (i.e., rod-like "balancers" on either side of the head of the larvae still present, and three distinct digits per forelimb; Harrison, 1969) were captured in each of the three lakes (n = 63), by dip-netting along the shore on July 28, 2019 (D0). Individuals were measured, swabbed (see below) and distributed in the individual mesocosms as follows: a third of the larvae remained in their lake of origin as controls, and the rest were translocated equally between the two other lakes (Figure 1b). Fifteen days later (D15), all the surviving larvae were swabbed again, and brought back to their lake of origin, where they were set free (n = 48). None of the larvae reached metamorphosis during the course of the experiment.

All the equipment was disinfected with a 3% solution of Virkon®S (DuPont) after each use. Our protocol was conducted with

the approval of the Ontario Ministry of Natural Resources (licence no. 1093589) and of Laurentian University's Animal Care Committee (protocol no. 2019-02-01).

2.2 | Sample collection

Our data collection consisted of a noninvasive skin swabbing. Each larva was held with a new pair of gloves and gently rubbed with a sterile swab (MW100 rayon tipped dry swab, MWE), five times back and forth on each side of the flanks (n = 63 + 48 = 111 samples in total). Three environmental control samples were collected per lake (n = 9 samples in total), by stirring swabs 20 times in the water. All swabs were preserved dry, on ice during sampling in the field, at −25°C upon arrival in the Algonquin Wildlife Research Station facilities, and at −80°C after being transported to our research laboratory, until further processing.

2.3 | DNA extraction

DNA was extracted from the swabs using the DNeasy PowerSoil Pro kit (Qiagen), following the manufacturer's instructions and including nontemplate controls (NTCs). The concentration and purity of each DNA extract was measured in an A_{260} absorbance assay using a Synergy H1 spectrophotometer (BioTek).

2.4 | Pathogen screening

Part of the DNA extracted from each swab was used to test the presence of ranaviruses and chytrid fungi. Although ranaviruses are identified primarily from tissue samples, the use of swabs is also a reliable noninvasive sampling method to detect them in amphibians (Ford et al., 2022; Gray et al., 2012; Standish et al., 2018). To avoid competition for reagents in case pathogen loads were very different (Thomas et al., 2018), we ran simplex assays for each pathogen,
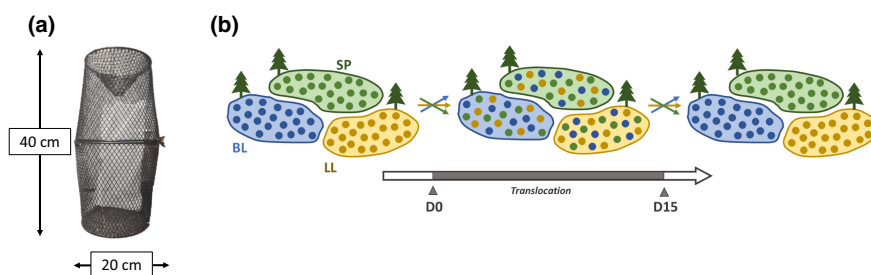
FIGURE 1 Experimental design. The larvae were individually maintained in custom-made mesocosms, consisting of a nylon bag stitched inside a minnow trap, filled with a handful of substrate and submerged to two thirds of its volume. The mesh of the nylon (2 mm ø) allowed typical prey to pass through, and thus the larvae could feed on them (a). The experimental plan consisted in capturing and skin-swabbing 21 larvae per lake (each larva is represented by a single dot) in three lakes of interest (BL, Bat Lake; LL, Lost Ray Lake; SP, Speckled Trout Lake), and then proceeding with a reciprocal translocation: for each lake of origin, a third of the larvae remained as controls, and the rest was equally divided and moved to the two other lakes. After 15 days, all individuals were swab-sampled again and then returned to their lake of origin, where they were set free (b).

following protocols to detect the MCP gene of ranaviruses (Leung et al., 2017), the ITS1 rRNA gene of *Bd* (Kriger et al., 2006) and the 5.8S rRNA gene of *Bsal* (Blooi et al., 2013). Because we expected low prevalence of chytrids, samples were pooled by five for *Bd* and for *Bsal* detection (Sabino-Pinto et al., 2019).

All reactions were run in 96-well plates, with triplicates of NTCs and of standards in five concentrations (1, 10, 10^3, 10^5 and 10^{10} genomic equivalents per microlitre). The positive controls were ordered as gBlocks (IDT) following a design by Standish et al. (2018). Samples were tested in duplicates, randomly assigned to different plates—in the event that results from duplicates were inconsistent, a third reaction was run and concluded from.

2.5 | Microbiota sequencing and bioinformatics

Library preparation (using primers 515F and 806R) and community amplicon sequencing of the hypervariable V4 region of the 16S rRNA gene (~254 bp) were conducted by Metagenom Bio on a MiSeq system (Illumina), at a depth of 30,000 reads. Demultiplexed sequences were processed using DADA2 version 1.8 (Callahan et al., 2016) managed through QIIME 2 version 2019.7 (Caporaso et al., 2010). Forward and reverse reads were truncated at decreasing quality (respectively 225 and 175 bp), and chimeric amplicon sequence variants (ASVs) were removed by reconstruction against more abundant parent ASVs. Taxonomy was assigned to representative sequences using a naive Bayesian classifier implemented in QIIME 2, trained against SILVA version 134, clustered at 99% identity (Pruesse et al., 2007). Assignments were accepted above a 0.7 confidence threshold. To identify symbiotic phylotypes with known inhibitory activity against *Bd* (referred to as *Bd*-inhibitory), representative sequences were aligned to the Antifungal Isolates Database (Woodhams et al., 2015) in QIIME 2.

Preprocessing of the sequences was carried out using the R package PHYLOSEQ (McMurdie & Holmes, 2013). Only bacterial sequences were kept, and contaminant ASVs identified from NTCs were removed using the R package DECONTAM (Davis et al., 2018). To address the uneven depth of coverage (different library sizes) across samples, we split the data set into a low-abundance data set (<20,000 reads per sample) in which samples were not normalized but were deleted if they contained fewer than 200 ASVs, and a high-abundance data set (>20,000 reads per sample) for which all samples were normalized at 20,000 reads by rarefaction without replacement (Cameron et al., 2021). Spurious ASVs making up <.001% of the total reads were filtered out from the data (Bokulich et al., 2013). Eleven salamander samples were deleted through the rarefaction process, and the final data set therefore comprised 7942 ASVs across a total of 100 salamander samples, and 1643 ASVs across a total of nine water samples.

2.6 | Statistical analysis

Statistical analyses were conducted in the R environment version 4.1.0 (R Core Team, 2022). All tests described below were conducted both on whole bacterial communities and on a subset restricted to *Bd*-inhibitory phylotypes only. Estimates associated with the covariates in all models were deemed significant if associated with a *p*-value below a .05 threshold.

Alpha diversity (within-sample diversity) was quantified using Chao1 (estimated ASV richness) and Shannon (estimated community diversity) indexes, and was investigated using linear models. Briefly, a model including the location and the total body length of the larvae as fixed effects was used to determine whether these factors influenced the alpha diversity of the microbiota samples collected before the translocation (D0). This model showed that body length (mean $\pm$ *SE* = 41.58 $\pm$ 1.19 mm) had no effect on the alpha diversity of the microbiota, and therefore this variable was excluded from subsequent models for parsimony purposes. To determine whether ontogeny or environmental change had a stronger effect on the structure of the skin microbiota, alpha diversity indexes were used as response variables in mixed models that included time of sampling (D0, D15) and translocation status (translocated, not translocated) as binary fixed effects, and initial location (D0) and identity of the larvae as random effects. Lastly, to investigate the resilience of the microbiota to host translocation, alpha diversity indexes measured at the end of the experiment (D15) were used as response variables in mixed-models including initial alpha diversity metrics of the same individuals (D0) and their translocation status as fixed effects, and their initial location (D0) as a random effect. The estimates associated with the effect of each covariate in these models were tested using ANOVAs. If the residuals of the models did not meet the assumptions of normality and of homoscedasticity associated with ANOVAs, log-transformations or nonparametric tests (Kruskal–Wallis [KW] and Wilcoxon-rank-test, followed by Dunn post hoc tests when necessary) with Bonferroni correction were used to investigate the effect of the corresponding covariates separately.

Beta diversity was calculated as the weighted Unifrac distance (Lozupone et al., 2011), and was visualized using principal coordinates analysis (PCoA), built using the R package VEGAN (Oksanen et al., 2017). Permutational multivariate analyses of variance (PERMANOVAs) implemented using the adonis function (*n* = 9999 permutations) were used to test similar models as for the alpha diversity. Thereby, the effect of site location on the similarity of bacterial communities was tested among initial samples (D0); the influence of time and translocation status on the community structure of all microbiota samples was tested with a model including initial location (D0) as a block; and the resilience of the microbiota to translocation was investigated among final samples (D15) with a model including translocation status as a fixed effect and initial location as a block. When suitable, pairwise differences between the levels of the covariates included in the PERMANOVAs were tested using a pairwise adonis test. Differences in within-group variation were investigated using a betadisper test. Features differing in abundance between initial samples (D0) and control samples (D15), as well as between translocated and control larvae (D15), were identified using DESEQ2 on unrarefied data (Love 2014). Shared ASVs between populations were visualized using Venn diagrams created in the R

package GGVENNDIAGRAM (Gao et al., 2021). Other graphical representations were plotted using the R packages GGPLOT2 (Wickham, 2016) and GGPUBR (Kassambara, 2019).

Lastly, we investigated potential drivers of mortality among the larvae. A logistic regression (generalized linear model [GLM]) was conducted with survival as a binary response, and body length, initial alpha diversity, and interaction between initial (D0) and final (D15) location as covariates. A pairwise adonis test was used to investigate potential initial differences in community structure (D0) between the microbiota of larvae that eventually died and that of individuals which survived at the end of the experiment (D15). Features differing in abundance between these two latter groups were identified using DESEQ2 on unrarefied data, separately for each of the three populations of salamander larvae. We performed BLAST searches against the NCBI database (www.ncbi.nlm.nih.gov) to obtain more information on these differentially abundant features.

3 | RESULTS

3.1 | Natural range of spatial variation in the skin microbiota of spotted salamander larvae

Each of the three lakes and of their salamander larvae populations initially (D0) harboured distinct microbiota. The total number of ASVs in water samples varied between lakes, with three times as many total ASVs in the microbiota of LL as in BL (Appendix S1–S10A). The total number of phylotypes also varied between salamander larvae populations, but with more total ASVs and more *Bd*-inhibitory ASVs in individuals from BL than in the other lakes (Appendix S2). Water samples were dominated by Proteobacteria, Actinobacteria and Acidobacteria (Appendix S1–S10B). Similarly, the skin microbiota of all larvae was dominated by Proteobacteria (89.5%), but the second most-abundant phyla differed between populations, being Bacteroidetes (5.8%) in larvae from BL, Firmicutes (3.5%) in larvae from LL and Verrucomicrobia (13.5%) in larvae from SP (Figure 2). Moreover, the relative abundance of phylotypes with

known inhibitory activity against *Bd* was higher in the microbiota of larvae from SP (18%) than from BL (12.5%) or LL (10.8%). Taken together, Proteobacteria and Bacteroidetes accounted for over 99.5% of the *Bd*-inhibitory phylotypes in the microbiota of the larvae at the beginning of the experiment (Figure 3).

Although the alpha diversity of the water's microbiota did not significantly differ between the three lakes (Chao1, ANOVA: $F = 1.29$, $df = 2$, $p = .343$; Shannon, ANOVA: $F = 3.29$, $df = 2$, $p = .108$), bacterial community richness of the skin microbiota of salamander larvae initially differed among populations (KW: $\chi^2 = 13.07$, $df = 2$, $p = .001$; Figure 4a). Post hoc Dunn tests showed that individuals from LL had significantly poorer communities than individuals from BL (Dunn: $z = -2.62$, $p = .013$) and SP (Dunn: $z = -3.44$, $p = .001$), while these latter had similar richness (Dunn: $z = 1.02$, $p = .465$). However, alpha diversity including abundance (measured with the Shannon index) did not differ between the three populations (ANOVA: $F = 1.41$, $df = 2$, $p = .252$; Figure 4b). More specifically, the alpha diversity of the *Bd*-inhibitory bacterial communities present within the microbiota of the larvae did not differ across sites (Chao1, KW: $\chi^2 = 5.68$, $df = 2$, $p = .058$; Shannon, ANOVA: $F = 2.19$, $df = 2$, $p = .122$; Appendix S3). Among these initial microbiota samples, alpha diversity was not influenced by the body size of the larvae (Chao1, Pearson: $t = -0.85$, $df = 55$, $p = .399$; Shannon, ANOVA: $F = 1.79$, $df = 1$, $p = .187$).

The microbiota of the water from the three sites differed strongly in beta diversity (PERMANOVA: $F = 3.03$, $R^2 = .50$, $df = 2$, $p = .046$) because of significant differences in the abundance of several ASVs (Appendix S1–S10C), but did not vary in compositional variance (Betadisper: $F = 0.51$, $df = 2$, $p = .570$). The beta diversity of initial skin bacterial communities differed between all salamander larvae populations, with the location of the individuals explaining over 17% of the variation in composition among initial microbiota samples (PERMANOVA: $F = 5.68$, $df = 2$, $p < .001$). Nevertheless, the compositional variance of the microbiota was homogenous across the three populations (Betadisper: $F = 0.56$, $df = 2$, $p = .578$). Similarly, the composition of *Bd*-inhibitory bacterial communities was significantly different across populations, as location explained 20% of the variation in beta diversity across samples (PERMANOVA: $F = 6.86$,
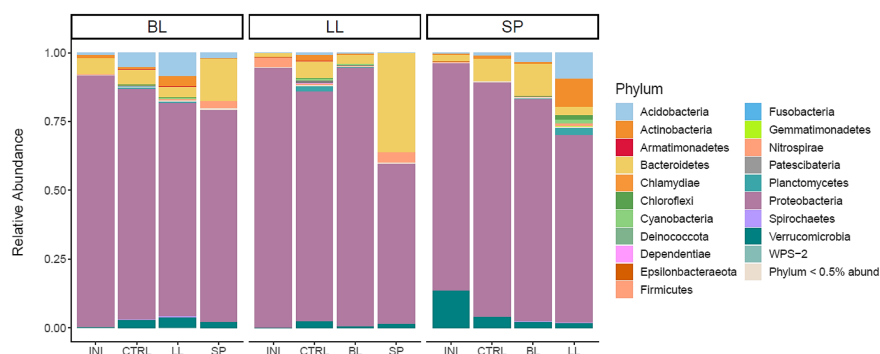
FIGURE 2 Mean relative abundance of the most frequent bacterial phylotypes in the skin microbiota of yellow-spotted salamander larvae, at the phylum level. Samples are grouped according to their experimental status (INI, initial sampling at D0; CTRL, control samples at D15; BL, transferred samples originating from BL; LL, transferred samples originating from LL; SP, transferred samples originating from SP) and sampling location (BL, Bat Lake; LL, Lost Ray Lake; SP, Speckled Trout Lake). Dominant phyla are identified in the key.
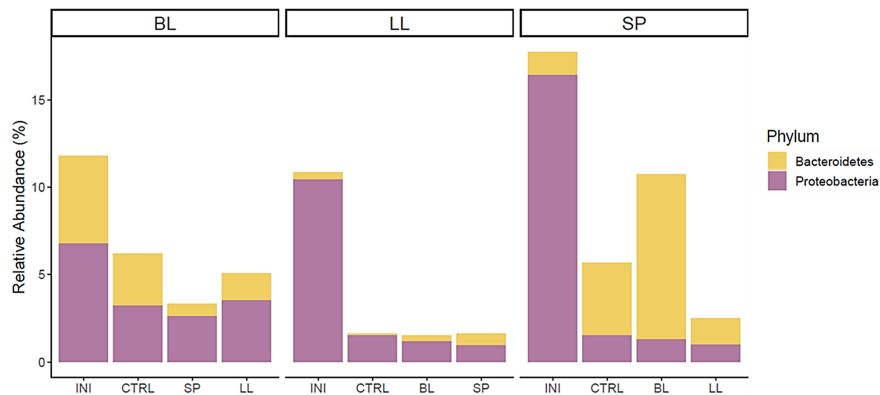
FIGURE 3 Mean relative abundance of the main phylotypes with known *Bd*-inhibitory activity in the skin microbiota of yellow-spotted salamander larvae, at the phylum level. Samples are grouped according to their experimental status (INI, initial sampling at D0; CTRL, control samples at D15; BL, transferred samples originating from BL; LL, transferred samples originating from LL; SP, transferred samples originating from SP) and sampling location (BL, Bat Lake; LL, Lost Ray Lake; SP, Speckled Trout Lake). Dominant phyla are identified in the key.
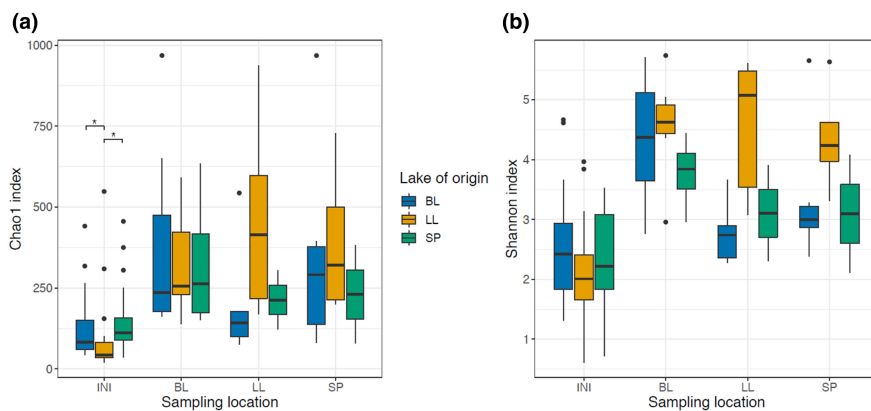
FIGURE 4 Alpha diversity of the skin microbiota of yellow-spotted salamander larvae at the beginning (D0, "INI") and the end (D15, by final location: BL, Bat Lake; LL, Lost Ray Lake; SP, Speckled Trout Lake) of the experiment, measured as Chao1 (a) and Shannon (b) indexes. The colour code indicates the initial site from where the larvae were collected, at D0. Brackets with asterisks indicate initial significant differences in richness between samples from LL and samples from the two other sites.

$df = 2$, $p < .001$). The compositional variance of *Bd*-inhibitory bacterial communities was significantly higher in larvae from BL than within individuals from LL (Betadisper: $F = 6.32$, $df = 2$, $p = .002$; Appendix S4).

3.2 | Relative effects of temporal variation and habitat-translocation on the skin microbiota of spotted salamander larvae

Both ontogeny and the translocation event influenced the composition of the skin microbiota of the larvae. Indeed, while Proteobacteria consistently constituted the majority of phyla at both D0 and D15, the second most abundant phyla varied between these time points. Moreover, translocated larvae had a different microbiota from that of control larvae maintained at the same location (Figure 2; Appendix S5). The relative abundance of *Bd*-inhibitory phylotypes in the microbiota of the larvae was differentially affected by the translocation, and decreased through time—although it remained lower

in individuals from LL (2%) compared to individuals from BL (7%) or SP (6%; Figure 3).

Regardless of their location, the microbiota of the larvae became more diverse within the 15 days of monitoring (Figure 4). Ontogeny had a strong and significant effect on the alpha diversity of the microbiota (Chao1 ANOVA: $F = 33.33$, $df = 1$, $p < .001$; Shannon ANOVA: $F = 45.95$, $df = 1$, $p < .001$), while habitat translocation did not (Chao1 ANOVA: $F = .72$, $df = 1$, $p = .398$; Shannon ANOVA: $F = 2.76$, $df = 1$, $p = .100$). In fact, the alpha diversity of samples collected from larvae at D15 was not different between control and translocated individuals (ANOVA: Chao1, $F = .33$, $df = 1$, $p = .570$; Shannon, $F = 2.00$, $df = 1$, $p = .167$), and was not affected by initial (D0) measures of alpha diversity (ANOVA: Chao1, $F = 2.31$, $df = 1$, $p = .138$; Shannon, $F = 0.89$, $df = 1$, $p = .353$). Similarly, the alpha diversity of *Bd*-inhibitory phylotypes increased significantly through time (Chao1 ANOVA: $F = 4.32$, $df = 1$, $p = .041$; Shannon ANOVA: $F = 18.81$, $df = 1$, $p < .001$) but was not affected by the translocation (Chao1 ANOVA: $F = 1.58$, $df = 1$, $p = .211$; Shannon ANOVA: $F = 2.96$, $df = 1$, $p = .088$; Appendix S3). Models restricted to D15

data confirmed that the alpha diversity of *Bd*-inhibitory communities was not different between control and translocated individuals (ANOVA: Chao1, $F = 0.78$, $df = 1$, $p = .384$; Shannon, $F = 1.88$, $df = 1$, $p = .179$), and also was not affected by initial (D0) measures of alpha diversity (ANOVA: Chao1, $F = 0.06$, $df = 1$, $p = .810$; Shannon, $F = 0.01$, $df = 1$, $p = .908$).

Ontogeny was also a stronger driver of host microbiota structure compared to the environment: the time difference between the two sampling events (D0 and D15) explained over 13% of the variation in beta diversity among all microbiota samples (PERMANOVA: $F = 15.34$, $df = 1$, $p < .001$) whereas their translocation status explained less than 0.9% (PERMANOVA: $F = 1.02$, $df = 1$, $p = .388$; Appendix S6). More specifically, at D15, control and translocated individuals had similar microbiota structure (PERMANOVA, $F = 1.11$, $R^2 = .03$, $df = 1$, $p = .318$; Figure 5) and compositional variance (Betadisper: $F = 1.90$, $df = 1$, $p = .174$). Analysis of the full data set also showed that the compositional variance of microbiota was not affected by ontogeny (Betadisper: $F = 1.24$, $df = 1$, $p = .263$). Similar patterns were observed among protective phylotypes: ontogeny explained over 17% of the variation in beta diversity of *Bd*-inhibitory bacterial communities among all microbiota samples (PERMANOVA: $F = 21.43$, $df = 1$, $p < .001$) whereas their translocation status explained less than 2% (PERMANOVA: $F = 2.10$, $df = 1$, $p = .099$). Analyses restricted specifically to D15 samples confirmed that the beta diversity of *Bd*-inhibitory communities was not affected by the translocation (PERMANOVA, $F = 2.17$, $R^2 = .05$, $df = 1$, $p = .104$). Moreover, the compositional variance of the protective microbiota was not affected by time (Betadisper: $F = 0.10$, $df = 1$, $p = .761$) nor by the translocation (Betadisper: $F = 0.05$, $df = 1$, $p = .837$).

Differential analyses revealed that the variations in community structure of control samples between D0 and D15 were driven by changes in abundance of 51 phylotypes, the majority belonging to Proteobacteria, but also to Bacteroidetes and Verrucomicrobia. In contrast to older samples (D15), the initial skin microbiota of the larvae (D0) showed significantly higher abundances in a bacterium from the phylum Verrucomicrobia, and in 11 genera of Proteobacteria (Appendix S7). Among *Bd*-inhibitory phylotypes, 10 ASVs, all belonging to Proteobacteria, were significantly more abundant within initial samples (D0; Appendix S8). When comparing the microbiota of control and translocated larvae at D15, differential analyses revealed significant differences in the abundance of 13 phylotypes, belonging to at least five different bacterial genera from the phyla Acidobacteria, Bacteroidetes, Proteobacteria and Verrucomicrobia. In contrast to control individuals, translocated larvae had a microbiota enriched in these taxa (Figure 6). However, we did not find any *Bd*-inhibitory phylotypes that differed significantly in abundance between control and translocated larvae.

3.3 | Larval mortality and disease screening in Algonquin Provincial Park, Canada

Quantitative polymerase chain reactions (PCRs) revealed that all samples were clear from ranaviruses and chytrid fungi. Nevertheless, 24% of the larvae died during the experiment (at D15: $n_{BL} = 3$; $n_{LL} = 2$; $n_{SP} = 10$ dead individuals). Survival was not influenced by the body length of the larvae (GLM: $\beta = 0.07$, $df = 1$, $p = .354$), nor by their translocation (GLM: $\beta = -0.63$, $df = 1$, $p = .496$), or their
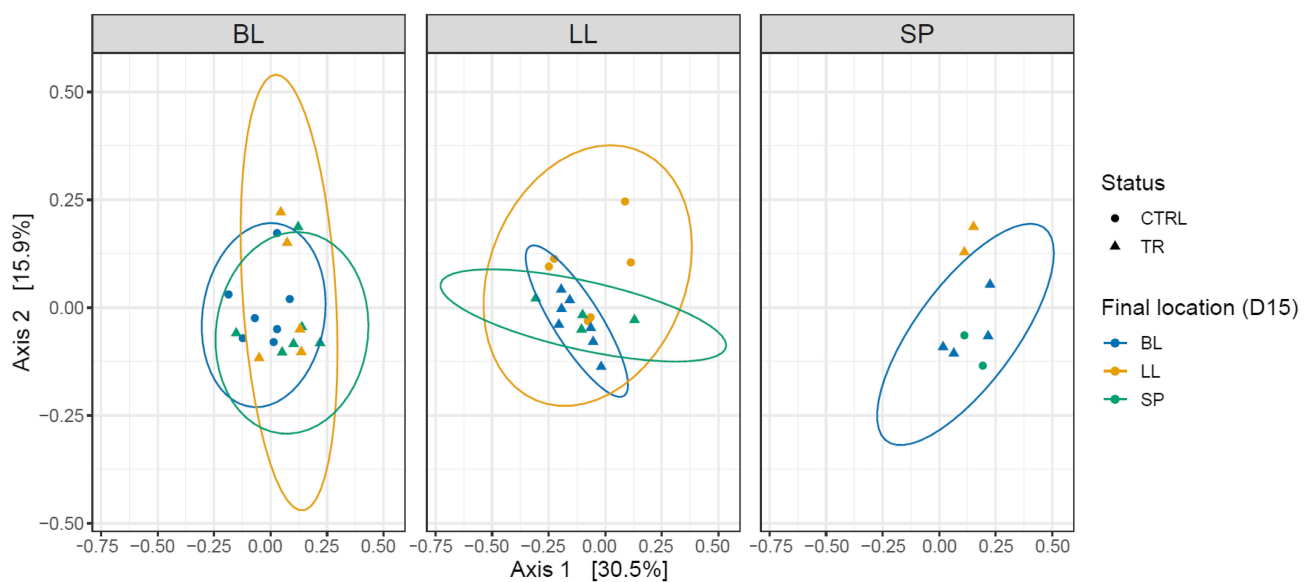
FIGURE 5 PCoA representing the beta diversity (calculated as the weighted Unifrac distance) of the skin microbiota of control vs. translocated yellow-spotted salamander larvae, 2 weeks after the translocation (D15). Results are plotted separately in three blocks to gather larvae initially coming from the same location (D0). The shape of the datapoints indicates whether they correspond to microbiota of control or translocated larvae (Status). The colour code and ellipses indicate the final location of the larvae at D15 (BL, Bat Lake; LL, Lost Ray Lake; SP, Speckled Trout Lake). Because of high mortality in larvae originally collected in SP, the number of samples of translocated larvae into LL and of control larvae that remained in SP (D15) was too limited to infer ellipses (right panel).

FIGURE 6 Significant $\log_2$ fold difference in abundance of bacterial phylotypes in the skin microbiota of translocated yellow-spotted salamander larvae 15 days after their translocation, compared to the microbiota of resident control larvae, that always remained in the same lake (D15). Each point represents a phylotype differentially abundant between translocated and control larvae. The colour code and the columns respectively indicate the phylum and the genus to which each of these phylotypes belongs. Genera that could not be identified are indicated as NA.
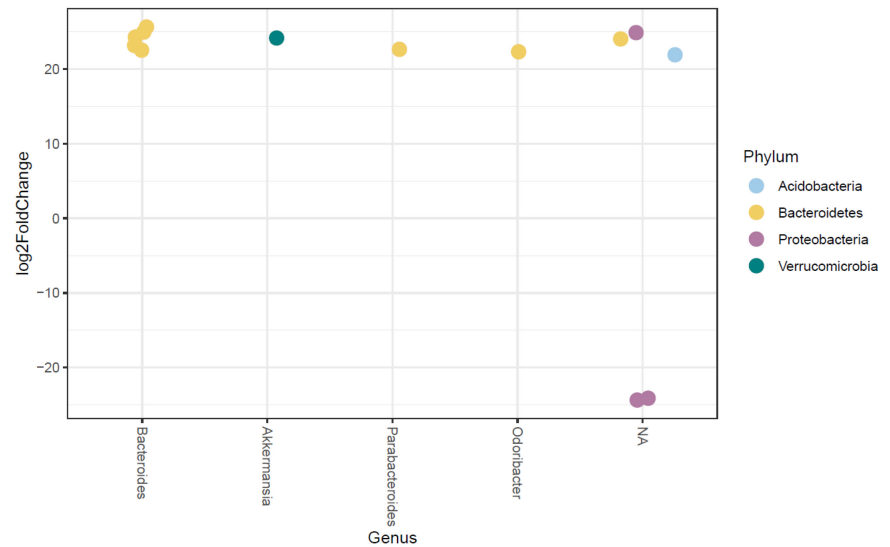
TABLE 1 Summary of logistic regression investigating the determinants of survival among the larvae at D15.

	Estimate	SE	z-value	p-value
(Intercept)	2.11	3.15	0.67	.5030
Initial body length	0.07	0.08	0.93	.3538
Initial alpha diversity	−0.47	0.43	−1.10	.2730
Status (TR)	−0.63	0.92	−0.68	.4957
Site of origin (LL)	−1.63	1.29	−1.26	.2073
Site of origin (SP)	−2.68	1.08	−2.48	**.0133**
Final site (LL)	−1.32	1.07	−1.23	.2179
Final site (SP)	−1.99	1.03	−1.93	.0531

Note: The estimates are given with their standard error (*SE*). The contrasts used for each categorical predictor are in parentheses. Initial body length and initial alpha diversity (measured with the Shannon index) were measured at D0. The effect of translocation (TR) was contrasted against control larvae at D15. The effects of the sites of origin (D0) and of final sampling (D15) were contrasted against BL (BL, Bat Lake; LL, Lost Ray Lake; SP, Speckled Trout Lake). The significant *p*-value is in bold type.

initial alpha diversity (GLM: $\beta = -0.48$, $df = 1$, $p = .273$), but it was influenced by their location of origin: larvae that were collected in SP had significantly lower chances of survival (GLM: $\beta = -2.68$, $df = 2$, $p = .013$), and the odds of surviving were 15.9 times higher for larvae originating from other lakes (Table 1). We found no differences in initial community structure (D0) between the larvae that eventually died and those which survived at D15 (PERMANOVA: $F = 0.12$, $df = 1$, $p = .147$).

However, differential analyses revealed that among larvae from BL, the microbiota of individuals that died before the end of the experiment was initially (D0) less abundant in 15 ASVs, all belonging to Proteobacteria, than that of larvae that survived (Appendix S9). More specifically, within the initial *Bd*-inhibitory microbiota of larvae from BL, two biomarkers (*Pseudogulbenkiania* sp. and *Deefgea* sp.) were significantly less abundant in larvae that eventually died compared to larvae that survived (Appendix S10). Among larvae

from LL, individuals that died were initially poorer in two ASVs, also belonging to the phylum Proteobacteria. Although their phylogenetic assignment using SILVA was limited to the family level (Enterobacteriaceae), BLAST searches suggested these two ASVs may be *Klebsiella pneumoniae* and *Salmonella enterica*. Among larvae from SP, individuals that died had a microbiota initially more abundant in two ASVs; BLAST searches suggested that one of these biomarkers may belong to the genus *Acidovorax* (Appendix S9).

4 | DISCUSSION

This study adds to our understanding of the organization of bacterial assemblages on the amphibian skin and expands our knowledge on the effects of anthropogenic environmental disturbances, such as conservation interventions, on animal microbiota. This is of particular importance for amphibians considering the key roles of their skin symbionts in their resistance to deadly skin diseases (Rebollar et al., 2020; Vredenburg et al., 2011). Taken together, our results suggest that population translocations conducted at an early life stage have a limited impact on the skin microbiota of amphibians. Moreover, the microbiota of salamander larvae seems to be more strongly shaped by ontogeny than by their environment. Although these observations should be confirmed in other amphibian models, our findings support population translocations as a promising strategy for amphibian conservation.

4.1 | Patterns of spatial and temporal variation in a naturally dynamic microbiota

The skin microbiota of yellow-spotted salamander larvae was dominated by Proteobacteria, with important proportions of Bacteroidetes, Firmicutes and Verrucomicrobia, as reported in many other amphibian species (Harrison et al., 2019; Kueneman et al., 2014; McKenzie et al., 2012; Sanchez et al., 2017). It was

different from that of the water, thus confirming the selectivity of the amphibian skin (Walke et al., 2014), and varied in composition, richness and beta diversity throughout locations. This was expected considering the high sensitivity of the skin microbiota of amphibians to environmental factors, which can drive major differences even among individuals from the same species (Bird et al., 2018; Varela et al., 2018). Indeed, despite similar alpha diversity, the water from the three lakes in the study had a distinguishable microbiota structure, as suggested by their strong variation in beta diversity.

In addition to this spatial diversity, we report a rapid shift in the organization of the skin microbiota of salamander larvae throughout their development. Community richness and diversity increased rapidly between stage 45 (D0) and the last stages before metamorphosis (D15). Ontogeny is known to affect the gut and mouth microbiota structure and diversity in frog larvae, but this is the first report of a change in such a narrow time frame (Griffiths et al., 2018; Warne et al., 2017). We identified many phylotypes that increased significantly in abundance between D0 and D15, including several *Acinetobacter* sp. and *Pseudomonas* sp. Many species in these genera are reported to inhibit chytrid fungi growth in vitro (Muletz-Wolz et al., 2017); thus, the developmental window between stage 45 and metamorphosis could be key in establishing host defences against pathogens.

Interestingly, our results show that the initial alpha diversity of individual microbiota does not affect their alpha diversity 15 days later. The important restructuring of bacterial communities over this short time window could be caused by a low selectivity of the larval skin at developmental stage 45, or by an increasing availability of ecological niches as the larvae grow—although we found no link between larval body length and alpha diversity of the skin microbiota. This counterintuitive result could also be caused by the depletion of bacterial communities during the initial sampling, whereby swabbing may have prevented other species from colonizing the microbiota and/or removed phylotypes that would otherwise have been retained in the community at D15 (Fukami, 2015). Considering that studies on temporal variation of individual skin microbiota often use repeated sampling over short time windows, it could be particularly interesting to determine whether they do exactly represent natural variation of bacterial communities.

4.2 | Effects of the translocation on the composition and diversity of the skin microbiota

Our findings suggest that the translocation event affected the composition, but not the alpha diversity nor the community structure (beta diversity) of the skin bacterial communities of salamander larvae. The microbiota of translocated individuals probably retained bacteria from their environment of origin while also being colonized by species from the site of transfer, as suggested by the numerous phylotypes that were significantly more abundant in translocated individuals. Interestingly, several of these taxa belonged to genera generally associated with gut microbiota, such as *Akkermansia*

sp. (Belzer & De Vos, 2012), *Odoribacter* sp. and *Parabacteroides* sp. (Wan et al., 2022; Zhang et al., 2018). Structural effects of translocation on skin bacterial communities, with a global shift towards the new environment's microbiota while maintaining microbes from the environment of origin, are reported in longer-term studies and in a large array of hosts, from Tasmanian devils (Chong et al., 2019), to Atlantic salmon fry (Uren Webster et al., 2020) and hellbender salamanders (Nolan, 2020). However, several translocation experiments report that the alpha diversity and community structure of microbiota are entirely determined by the environment of destination of translocated individuals (Bletz et al., 2016; Uren Webster et al., 2020). In yellow-spotted salamander larvae, we found that the skin microbiota was very resilient to environmental change, as translocated individuals maintained microbiota of similar alpha and beta diversity as control larvae that remained in their lake of origin. This confirms that microbial colonization in early life can affect microbial assemblages in later stages (Robinson et al., 2010; Sanchez et al., 2017; Uren Webster et al., 2020), and is particularly important in the context of amphibian conservation, as the establishment of their microbiota during critical developmental windows may affect their later metabolism and susceptibility to diseases (Warne et al., 2019). However, our study only lasted 15 days in order to sample the larvae before metamorphosis, as they probably undergo tremendous changes in their microbiota when becoming terrestrial (Kueneman et al., 2014); it is therefore unknown whether structural changes in the microbiota of translocated individuals may have been recorded had they been monitored over a longer time window.

4.3 | Protective phylotypes, susceptibility to diseases and mortality

The proportion of *Bd*-inhibitory phylotypes in the microbiota of our experimental larvae was relatively low compared to other amphibian taxa (Walke et al., 2017). Considering that all individuals in the study were clear from chytrid fungi, this concurs with work from Walke et al. (2017) suggesting that selection for inhibitory phylotypes may be reduced in populations associated with low pathogen prevalence. In comparison with our results, different patterns of microbiota restructuring throughout ontogeny or translocation would probably be expected in locations with a higher prevalence of ranaviruses and chytrid fungi. Regardless, note that most of the phylotypes identified within our samples remained untested against *Bd* (74% at D0 and 70% in control samples at D15), and that no database was available to test their activity against the many other pathogens that threaten amphibians (Pessier, 2014) and induce changes in their skin microbial communities (Federici et al., 2015; Harrison et al., 2019). Moreover, the production and function of microbial secondary metabolites can differ among pathogen isolates (Antwis & Harrison, 2018), so 16S sequencing is not sufficient to ascertain the antifungal properties of the identified phylotypes. Investigating bacterial inhibitory activity against a broader range of emerging diseases is therefore urgently needed to provide researchers and conservation stakeholders with

more exhaustive databases. Nonetheless, the quantification of *Bd*-inhibitory bacteria provides important insight into the potential trends followed by symbionts with protective activity and might be extrapolated to *Bsal*-inhibitory bacteria because the rare phylotypes with known activity against that latter fungus are also inhibitory against *Bd* (Woodhams et al., 2018).

Despite the absence of ranaviruses and chytrid fungi, mortality among our study animals was not unexpected since the larvae were very close to metamorphosis at the end of the experiment—a life period commonly associated with increased mortality in amphibians (Carey et al., 1999; Rollins-Smith, 1998; Werner, 1986). We found no link between microbiota diversity or structure and host mortality, but the larvae originating (D0) from the SP site were associated with significantly higher mortality rates than individuals from BL and LL. Interestingly, differential abundance analyses revealed that the larvae from SP that died before D15 had a microbiota initially (D0) enriched in an *Acidovorax* sp. Although this genus is found in the commensal microflora of other amphibians (Lauer et al., 2008), it is more prevalent in individuals infected with *Bd* (Federici et al., 2015) and within skin wounds (Hernández-Gómez et al., 2017). It is therefore conceivable that in larvae with elevated abundance of *Acidovorax* sp., the initial (D0) swabbing of their fragile epidermis may have caused skin lesions that were aggravated by this bacterium, thus causing the death of these individuals. Regardless, the translocation event itself was not a cause of mortality, which is encouraging for this conservation strategy. Among the populations from the two other sites, some of the less abundant phylotypes in larvae that died compared to larvae that survived are taxa commonly found in amphibians (e.g., *Salmonella enterica*; Srikantiah et al., 2004) or in freshwater lake sediments (*Pseudogulbenkiania* sp.; Weber et al., 2009), although surprisingly, one of these taxa (*Klebsiella pneumoniae*) is known to cause deadly infections in anuran models (Hallinger et al., 2020); this confirms the fact that the impact of each bacterial taxon on its host is also dependent on the rest of the microbial community.

5 | SUMMARY: VIEWING AMPHIBIAN CONSERVATION THROUGH A MICROSCOPIC LENS

Incorporating host-associated bacterial communities to conservation research is increasingly recognized as a promising avenue to ensure future viability of host populations (Trevelline et al., 2019; West et al., 2019). The contribution of the resident microbiota to essential functions for the health of its host, and their high interdependence, have led to the emergence of the "holobiont" concept (Carthey et al., 2020), recognizing the host and its associated micro-organisms as one functional unit (Esser et al., 2019; Morar & Bohannan, 2019; Zilber-Rosenberg & Rosenberg, 2008). In this context, we suggest that optimal life stages for population translocations should be chosen using a comprehensive approach, considering holobionts rather than hosts alone, to avoid disrupting key windows for the establishment of microbial communities. Our results are encouraging as

they suggest that in situ conservation approaches such as translocations do not affect the structure of the microbiota or the survival of salamander larvae, but future research should further investigate the long-term effect of these management strategies on amphibian microbiota, using various life stages and biotopes. Ultimately, shifting our current conservation practices towards management of the holobiont requires a better understanding of the dynamics of organization of animal microbiota but has the potential to increase conservation success of endangered hosts faced with the sixth mass extinction crisis (Carthey et al., 2020; Wake & Vredenburg, 2008).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data and code are publicly available at the Figshare repository (https://figshare.com/s/e7231492797fcff85fb3 and https://figshare.com/s/e7231492797fcff85fb3?file=38176905).

BENEFIT-SHARING STATEMENT

Our research addresses a priority concern, in this case the evaluation of amphibians (the most endangered vertebrate class on earth). While in the field, L.F.M. contributed to the education and training of several undergraduate students. Benefits from this research accrue

from the sharing of our data on a public database, as described above.

ORCID

Léa Fieschi-Méric 🅐 https://orcid.org/0000-0002-5196-8877
Pauline van Leeuwen 🅐 https://orcid.org/0000-0002-9558-1359
Mathieu Denoël 🅐 https://orcid.org/0000-0002-3586-8323
David Lesbarrères 🅐 https://orcid.org/0000-0002-5505-1935

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