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Disruption of skin microbiota contributes to salamander disease

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Escalating occurrences of emerging infectious diseases underscore the importance of understanding microbiome–pathogen interactions. The amphibian cutaneous microbiome is widely studied for its potential to mitigate disease-mediated amphibian declines. Other microbial interactions in this system, however, have been largely neglected in the context of disease outbreaks. European fire salamanders have suffered dramatic population crashes as a result of the newly emerged *Batrachochytrium salamandrivorans* (*Bsal*). In this paper, we investigate microbial interactions on multiple fronts within this system. We show that wild, healthy fire salamanders maintain complex skin microbiotas containing *Bsal*-inhibitory members, but these community are present at a remarkably low abundance. Through experimentation, we show that increasing bacterial densities of *Bsal*-inhibiting bacteria via daily addition slowed disease progression in fire salamanders. Additionally, we find that experimental-*Bsal* infection elicited subtle changes in the skin microbiome, with selected opportunistic bacteria increasing in relative abundance resulting in septicemic events that coincide with extensive destruction of the epidermis. These results suggest that fire salamander skin, in natural settings, maintains bacterial communities at numbers too low to confer sufficient protection against *Bsal*, and, in fact, the native skin microbiota can constitute a source of opportunistic bacterial pathogens that contribute to pathogenesis. By shedding light on the complex interaction between the microbiome and a lethal pathogen, these data put the interplay between skin microbiomes and a wildlife disease into a new perspective.

1. Introduction

Advances in the knowledge of symbiotic microbiomes are changing our understanding of vertebrate host biology and ecology [1,2]. Resident microbiotas of metazoans are intricately linked to host health, whether it be through participation in energy metabolism, immune system development, or contributing to defence against pathogens [1,3–6]. Symbiotic microbes can occupy a central role in host–pathogen interactions, eliciting protective effects against invading pathogens through space and nutrient competition, production of anti-pathogen compounds as well as immuno-modulatory stimulation [4,7]. Elucidating the role of the microbiota within host–pathogen systems is critical for understanding the ecology of the disease.

Pathogenic fungi are unprecedented players in emerging infectious diseases [8]. From plants to vertebrates, infectious diseases caused by fungi are threatening food resources and leading to biodiversity loss [8–10]. Wheat stem rust and rice blast disease threaten important crops upon which humans depend (*Puccinia graminis*, *Magnaporthe oryzae* [11]). Bees responsible for pollination

are being devastated by colony collapse disorder (*Nosema* species [12]). Bat populations in North America are collapsing due to White-nose syndrome (*Geomyces destructans* [13]). Furthermore, amphibian chytridiomycosis, originally caused by the sole fungus, *Batrachochytrium dendrobatidis* [14,15], has been marked as a main culprit of amphibian declines within the so-called sixth mass extinction [16]. This cutaneous pathogen is considered the largest disease threat to the world's biodiversity as it has ravaged amphibian communities globally [16,17].

The recent emergence of a second amphibian-infecting chytrid, *Batrachochytrium salamandrivorans* (*Bsal* [18]) adds to the disease threat to these animals. *Bsal* poses a significant threat to western Palearctic salamanders, and, in particular, is responsible for severe declines of European fire salamanders, *Salamandra salamandra* [19,20]. *Bsal* invades keratinized amphibian skin, leading to superficial erosions and numerous deep ulcerations across the body of susceptible host species [18]. As infection escalates and induces chytridiomycosis it can result in death in less than one month [18–20]. Salamander hosts appear to have little ability to fight back against *Bsal* through host-based defences. Expression of immune genes remained unchanged during experimental infection of an Asian species, *Tylosotriton wenxianensis* [21]. Furthermore, fire salamanders, *S. salamandra*, mounted no immune response after five cycles of exposure-clearance regimes [20].

To date, limited work has been conducted on the role of host microbiota in *Bsal*-infection dynamics [22], yet a thorough understanding of salamander-microbiome-*Bsal* interactions is clearly essential. It is plausible that resident skin microbiota contributes to the host's mucosal defences against *Bsal*, which would open new horizons for disease mitigation [23]. However, the massive destruction of the epidermis during *Bsal* infection may equally predispose opportunistic pathogens to cause fatal septicemia. These juxtaposing ideas raise the pivotal question: What is the role of skin bacteria in *Bsal* infection of fire salamanders? Are they a friend, foe or bystander?

Here, we combine evidence from field studies and laboratory experiments to understand the role of bacteria in *Bsal* infection dynamics of the highly susceptible fire salamander. We surveyed healthy populations of wild fire salamanders to determine their natural skin bacterial densities, and performed laboratory experiments to investigate the response of salamander skin microbiota to *Bsal* infection. We further evaluated the function of cultivable resident bacteria against *Bsal* and the ability of these bacteria to alter infection dynamics *in vivo*.

2. Methods

(a) Field sampling

Healthy, adult European fire salamanders (*Salamandra salamandra*) were sampled at multiple locations across Germany in 2015 (electronic supplementary material, tables S1 and S2). At the time of sampling, all amphibians screened for *Bsal* from these locations, including the fire salamanders in our study, were *Bsal*-negative [24]. Adults were captured with gloved hands and skin swabs were taken following standard methods [25,26] for either DNA-based analyses (qPCR estimation of bacterial abundance and 16S amplicon sequencing of bacterial community) or cultivation of skin microbes. Swabs for DNA-based analyses were stored dry, and swabs for cultivation were stored in 20% glycerol to maintain bacterial cell integrity.

All samples were stored in ice and immediately frozen upon return to the laboratory.

(b) Culturing of skin bacteria

Skin bacteria were cultured from fire salamander populations across Germany (electronic supplementary material, table S2). Culturable skin swabs were processed as explained in [26].

(c) *Bsal* growth inhibition assays

A total of 708 bacterial isolates from fire salamander skin were tested in *Bsal* growth inhibition assays. Assay methodology followed a modified version of the 96-well assay method described in [27] (see electronic supplementary material, Methods). Inhibitory function against *Bsal* was determined by comparison of *Bsal* growth rate in the presence of bacterial CFS with that of the nutrient-depleted control (*Bsal* zoospores grown without additional nutrients) with FDR corrections. Enhancing function against *Bsal* was determined by comparison of *Bsal* growth rate in the presence of bacterial CFS with that of the positive control (*Bsal* zoospores growth in TGH media) with FDR corrections. Selected bacteria (electronic supplementary material, table S3) were tested multiple times to explore functional consistency.

(d) Liver isolate cultivation

Bsal infection results in deep ulcerations of the skin surface. This breaching of the integrity of the skin barrier may result in bacterial invasion of internal organs and the blood. To investigate this, bacterial isolates from the livers of nine fire salamanders that died due to experimental *Bsal* infection and nine non-*Bsal*-infected salamanders were cultured on Columbia agar with sheep blood. Cultures were incubated at 15°C and isolated into pure culture. Morphologically distinct bacteria were identified using Sanger sequencing of the 16S rRNA gene.

(e) *Bsal* infection experiment for microbiome analysis

Twelve captive-bred fire salamanders (six control, six *Bsal*-exposed) were housed individually at $15 \pm 1^\circ\text{C}$ on moist tissue, with access to a hiding place (PVC pipe) and a water container. Salamanders were exposed to *Bsal* (AMFP13/01; 5000 zoospores ml^{-1}) by dripping 1 ml of a zoospore suspension onto the salamander. Controls received 1 ml of artificial pond water. Animals were fed twice weekly with crickets. Individuals were swabbed as described in Bletz *et al.* [25] prior to exposure and 10 days post-exposure.

(f) Bacterial addition experiments

The bacterial addition experiment was conducted to evaluate the function of bacteria in an *in vivo* context and to understand if bacteria on the skin can alter *Bsal*-infection dynamics. Bacterial isolates for addition experiments were selected from *in vitro* *Bsal* growth assay results. Selection criteria are outlined in the electronic supplementary material, Methods.

Twenty-six captive-bred fire salamanders were housed individually and fed as explained above for a duration of 11 weeks. The following treatments were used: (i) daily addition of a *Stenotrophomonas* sp. (*Bsal*-enhancing, $n = 7$), (ii) daily addition of a *Pseudomonas* sp. (*Bsal*-enhancing, $n = 7$), (iii) daily addition of a sham treatment of 1 ml of sterile water with an agar swab ($n = 7$), and (iv) no treatment ($n = 5$). Once the bacteria had grown on the agar plate, a swab was used to collect bacterial cells from the plate. The collected cells were then suspended in 10 ml of sterile artificial pond water. The sham treatment with the agar swab was included as a control for this process. Bacterial cells were quantified by spectrophotometry that was verified by CFU counts. Bacterial treatments were administered daily by adding 1×10^8 bacterial cells suspended in 1 ml of sterile artificial pond water.

Daily administration of treatments was completed to ensure the continual presence of these bacteria on the skin for the duration of the experiment (11 weeks/77 days). *Bsal* exposure was given as described above (except 1×10^4 zoospores ml^{-1}). Individuals were swabbed on day 0 prior to any treatment, on day 3 prior to *Bsal* exposure, and at weekly intervals following *Bsal* exposure for 11 weeks or until animals were removed from the experiment. Swabs were used to quantify bacterial load, *Bsal* load, and to culture skin bacteria for Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF) identity confirmation. Animals were removed from the experiment when clinical signs indicated lethal disease (high *Bsal* load and morbidity). At this time, skin swabs were taken, and individuals were subsequently euthanized with MS-222 overdose.

(g) DNA extraction

Bacterial DNA was isolated following protocols in previous studies: for microbiome analyses of field swabs, and the analysis of microbiome response in the *Bsal* exposure experiment, we used the MoBio PowerSoil DNA Isolation kit [25]; for skin bacterial isolates we used a Chelex protocol [26]; and for the bacterial addition experiment, Prepman was used [18]. MoBio extraction methods were used for samples where exploration of the microbial community composition was the goal. Prepman extraction was used in the bacterial addition experiment because it is more cost-effective and microbiome analysis was not being performed. Importantly, no comparisons were made among samples from different extraction methods.

(h) Identification of bacterial isolates

A fragment of the 16S rRNA gene from cultured isolates was PCR-amplified with the primers 27F (AGAGTTTGATCCTGGCT-CAG) and 907R (CCGTCAATTCMTTGTAGTTT). PCR products were sequenced at LGC Genomics in Berlin, Germany. Sequencing produced approximately 500–800 bp for each bacterial isolate. Sequences were cleaned in CodonCode Aligner, and aligned with PyNAST. Taxonomy was assigned with RDP in Quantitative Insights Into Microbial Ecology (QIIME) [28] and a phylogenetic tree was built with fasttree [29].

(i) Quantification of bacterial abundance and *Bsal*

Total bacterial abundance and *Bsal* infection intensities were estimated with qPCR. Total bacteria were quantified using a SYBR Green qPCR assay using the universal bacterial primers described in [30]. qPCR conditions were 10 min at 95°C, followed by 39 cycles of 60 s at 94°C, 60 s at 50°C, 60 s at 60°C, and a final elongation for 15 min at 60°C. Primer concentration was 0.5 μM . Within the experiments, bacterial densities were determined by calculating the surface area swabbed from measurements of the trunk length and width. *Bsal* infection intensities were determined using the qPCR assay described in [31]. qPCRs were performed using the CFX384 Bio-Rad detection system.

(j) 16S rRNA characterization of communities

16S rRNA gene amplicon sequencing was performed on field samples and on samples collected during the *Bsal* exposure experiment. The V4 region of the 16S rRNA gene was PCR-amplified with dual-indexed primers as described in [32]. Pooled amplicons were sequenced on an Illumina MiSeq with 2×250 paired-end technology at the Helmholtz Center for Infectious Research in Braunschweig, Germany. QIIME [33] was used to demultiplex and quality-filter the sequence data, and sequences were clustered into sub-operational taxonomic units (sOTUs) using Deblur [34]. Detailed methodologies for

processing the amplicon sequencing data are provided in the electronic supplementary material, Methods.

(k) MALDI-TOF

During the bacterial addition experiment, MALDI-TOF MS direct cell profiling was used to confirm the presence of the administered bacteria [35]. More specifically, bacteria were re-isolated from the skin of treated fire salamanders and MALDI-TOF was used to compare the profile of each morphologically distinct, re-cultured isolate to that of the original inoculum. MALDI-TOF was performed using an Autoflex Biotyper MALDI-TOF mass spectrometer (Bruker Daltonik) using the direct transfer method and α -cyano-4-hydroxycinnamic acid (HCCA) as a matrix, according to the manufacturer's guidelines. Detailed methodologies are provided in the electronic supplementary materials.

(l) Scanning electron microscopy

Scanning electron microscopy (SEM) of the skin of *Bsal*-free fire salamanders (3) was carried out to provide a visual assessment of the density of skin microbes. SEM was performed after 2% paraformaldehyde and 2.5% glutaraldehyde fixation in 0.1 M phosphate buffer [18].

(m) Statistical analyses

All statistical analyses were performed in R (v. 3.4, [36]) unless otherwise stated. For the *Bsal* exposure experiment looking at microbiomes, bacterial density and alpha diversity were analysed with general linear mixed effect models (GLMMs, lmer4; [37]). Treatment, time, and the time by treatment interaction were included in GLMMs along with individual as a random factor to account for repeated sampling. Data were normalized with log transformations as needed. We used ADONIS2 [38] to perform a permutational multivariate analysis of variance to assess whether time and *Bsal* exposure explained significant portions of the observed variation in microbial community structure (i.e. beta diversity). Time, treatment, and the time by treatment interaction were included as explanatory variables. We used Linear discriminant analysis effect size method (LEfSe) to identify differentially abundant bacterial taxa between the microbial communities of *Bsal*-exposed and control salamanders at the post-exposure time point [39].

In the bacterial addition experiment, all analyses of bacterial density and *Bsal* infection loads were performed on samples collected from the first 28 days of the experiment when all salamanders were still in the experiment. Bacterial density was statistically analysed with GLMMs as described above. To explore main effects and significant interactions we performed pairwise *post hoc* comparisons among treatments and time points as necessary. Rate of change in *Bsal* infection intensity was calculated as the slope of the estimated raw *Bsal* loads for each individual between day 14 (first time point where *Bsal* was detected) and 28 (last time point when all individuals were still in the experiment). These calculated rates were then compared across treatments with a Kruskal–Wallis test. Host survival was evaluated with a Cox log-rank test using the survival package [40]. Boxplot and survival graphs were created with ggplot2 and the survminer package [41].

3. Results

(a) Bacterial diversity and density on *Salamandra* skin

Skin bacterial communities from 275 wild, healthy fire salamanders in Germany were characterized using 16S amplicon

Figure 1. Natural densities of bacteria on the skin of fire salamanders. (a) Scanning electron microscopy image of salamander skin, showing a skin gland opening (arrow) and an overall lack of epidermal cell-associated bacteria. (b) Box plot showing qPCR estimates of bacterial abundance on fire salamander skin at four locations within the Eifel in western Germany. The upper and lower limits of the box represent the first and third quartiles, with the bold line representing the median. Whiskers extend to the minimum and maximum values, and points represent outliers.

sequencing. The richness of sub-operational taxonomic units (sOTUs) on individuals across populations averaged 212.6 ± 9.56 s.e. The skin microbiota predominantly comprised *Proteobacteria* (48.8%), *Bacteroidetes* (24.8%), *Actinobacteria* (8.2%), *Firmicutes* (6.7%), *Cyanobacteria* (4.1%), *Acidobacteria* (2.8%) and *Verrucomicrobia* (2.7%) (electronic supplementary material, appendix, figure S1). Furthermore, we found that microbial community structure differed across locations (PERMANOVA, Pseudo-F = 4.18, $p = 0.001$; electronic supplementary material, figure S2).

The average bacterial abundance on fire salamander skin ($n = 94$) across populations was $3.5 \times 10^4 \pm 6.7 \times 10^3$ s.e. rRNA copies/swab (figure 1b). The average bacterial abundance for the four populations was $2.34 \times 10^4 \pm 4.8 \times 10^3$ s.e., $1.43 \times 10^4 \pm 3.0 \times 10^3$, $2.63 \times 10^4 \pm 4.5 \times 10^3$ and $6.39 \times 10^4 \pm 1.3 \times 10^3$ for Fleischbach (Fi), Zweifallshammer (KAZ), Kallerbach (KB) and Solchbachtal (SO), respectively. A similarly low bacterial abundance on the skin surface was shown through SEM visualization, where often no or only a very limited number of bacterial cells can be seen (figure 1a).

(b) *Bsal* infection elicits subtle changes in *Salamandra* skin microbiome, which are associated with septicemic events

We characterized the cutaneous microbiome response to *Bsal* invasion using experimental infection of fire salamanders (*Bsal*-exposed: $n = 6$, control: $n = 6$) and 16S amplicon sequencing. *Bsal* infection had no effect on skin bacterial abundance, bacterial richness and diversity, or community structure. Average bacterial density on the salamander skin was 5.7×10^3 rRNA copies mm^{-2} (s.e. ± 1990.5) prior to exposure. There was no significant change as a result of *Bsal* infection or through time (GLMM: Treatment-F = 0.25, $p = 0.63$; Time-F = 0.63, $p = 0.45$; Interaction-F = 0.43, $p = 0.53$; figure 2a). Skin bacterial richness and diversity on fire salamanders before exposure averaged 142 ± 6.9 s.e., 81.7 ± 21.2 , and 15.7 ± 1.1 for sOTU richness, effective number of sOTUs (exp(Shannon Index)), and Faith's phylogenetic diversity

(PD), respectively. *Bsal* infection also had no significant effect on bacterial richness or diversity of skin communities; however, there was a significant increase through time in effective number of sOTUs (GLM: Effective number of sOTUs: Treatment-F = 0.02, $p = 0.89$; Time-F = 5.76, $p = 0.04$, Interaction-F = 1.35, $p = 0.27$; figure 2b; electronic supplementary material, figure S3). Bacterial community structure (i.e. beta diversity) also did not change as a result of *Bsal* infection, but significantly shifted through time (PERMANOVA: weighted Unifrac: Treatment-Pseudo-F = 0.67, $p = 0.72$; Time-Pseudo-F = 2.78, $p = 0.01$; Interaction-Pseudo-F = 1.016, $p = 0.374$; unweighted Unifrac: Treatment-Pseudo-F = 0.68, $p = 0.86$ Time-Pseudo-F = 1.73, $p = 0.03$; Interaction-Pseudo-F = 1.1119, $p = 0.325$; figure 2c).

While *Bsal* exposure elicited no significant change in alpha and beta diversity of fire salamander skin microbiota, particular bacterial taxa were found to be differentially abundant on infected versus control individuals using LEfSe. Seven bacterial sOTUs exhibited greater relative abundance on infected individuals, and three exhibited greater relative abundance on non-infected individuals at the post-exposure time point (figure 2d; electronic supplementary material, table S4). An *Aeromonadaceae* sp., a *Chryseobacterium* sp., a *Fusobacteriaceae* sp., a *Lactococcus* sp. and *Stenotrophomonas acidominiphila* were differentially associated with *Bsal*-infected salamanders, whereas an *Actinomycetales* sp., a *Dyadobacter* sp., a *Luicobacter* sp. and a *Pedobacter* sp. were more common on control salamanders.

Moreover, *Bsal*-induced chytridiomycosis resulted in septicemic events, likely resulting from the breaching of the skin barrier by *Bsal*. Forty-five bacterial isolates were cultured and successfully identified from livers of nine infected fire salamanders. These liver-colonizing bacteria were from three phyla: *Proteobacteria* (21 isolates) *Bacteroidetes* (20 isolates) and *Actinobacteria* (four isolates) (electronic supplementary material, table S5). Most notably there were 15 isolates identified as *Acinetobacter johnsonii* and nine isolates identified as *Chryseobacterium* sp. (electronic supplementary material, table S5). No bacteria were isolated from the livers of fire salamanders that were not infected with *Bsal*.

Figure 2. Response of fire salamander skin microbiota to *Bsal* infection. (a) qPCR estimates of bacterial density (rRNA copies/mm²), (b) species richness (sOTU richness) of skin bacterial communities and (c) bacterial community structure on control and *Bsal*-exposed salamanders. Bacterial community structure is visualized with Principal coordinate analysis of the weighted Unifrac distances. (d) Six representative sOTUs identified by LEfSe to be differentially abundant on *Bsal*-exposed individuals and control individuals.

(c) Increased density of *Bsal*-inhibitory bacteria dampens *Bsal* infection, but only marginally changes overall outcome

We evaluated the function of cutaneous bacteria on fire salamanders using *in vitro* culture-based approaches as well as *in vivo* experimentation. The cultured isolates ($n = 708$) were from the following phyla: *Actinobacteria* (44.6%), *Proteobacteria* (37.6%), *Bacteroidetes* (12.4%) and *Firmicutes* (5.4%) (figure 3a). We found that these resident skin bacteria exhibited a range of functional capacities against *Bsal*. Of the 708 isolates tested with *in vitro* growth assays, 30% inhibited *Bsal* growth, 13% enhanced *Bsal* growth and 57% had no effect (figure 3b). It is important to note, not all bacteria consistently exhibited the same function against *Bsal*. Re-testing of multiple skin bacterial isolates ($n = 17$) against *Bsal* resulted in variable functionality. This finding is potentially due to differences in the cell density of cultures when bacterial products were collected (electronic supplementary material, table S3).

Bacterial density at the start of the experiment (prior to experimental treatments) did not differ between experimental groups ($n = 7$ per treatment; $\text{KW-}\chi^2 = 0.7851$, $p = 0.853$). After bacterial addition began, bacterial density differed significantly among treatments (LMM, $F = 12.67$, $p < 0.001$) and through time (LMM, $F = 49.57$, $p < 0.001$) (figure 3c). There was also a significant interaction between time and treatment

(LMM, $F = 4.96$, $p = 0.003$). To explore these main effects and the interaction we performed pairwise *post hoc* comparisons among treatments and time points. Daily addition of bacteria to fire salamander skin in both the inhibitory and enhancing treatment increased bacterial density in comparison to the no-treatment control (pseudo: $t = -3.95$, $p < 0.001$; steno: $t = -5.23$, $p < 0.001$), however, not in comparison to the agar-wash control (pseudo: $t = 2.12$, $p < 0.152$; steno: $t = 0.69$, $p = 0.89$). The mechanism for bacterial increase in agar-treated animals remains unclear. It is possible, for example, that the minimal agar media present in the inocula promoted bacterial growth; alternatively, the dilution of host skin factors could remove inhibition; to decipher this future work could consider including a water-only treatment control. Furthermore, this effect diminished over time; by day 21 there were no differences in bacterial density among treatments (KW $\chi^2 = 1.95$, $p = 0.58$). Re-isolation success of the added bacteria to confirm their presence on the skin proved highly inconsistent through time on a given individual. Nevertheless, MALDI-TOF identification of re-isolated bacteria confirmed the presence of the administered bacteria on the skin of 12 of 14 individuals on at least one time point throughout the experiment (electronic supplementary material, table S6).

The artificial addition of selected bacteria to salamander skin affected *Bsal* infection dynamics and survival. There was some evidence that the rate of increase in *Bsal* infection

Figure 3. Phylogenetic distribution and function of cultured skin bacteria from all sampled salamanders and the effects of bacterial addition on *Bsal*-infection dynamics. (a) Phylogenetic distribution of skin bacterial isolates. The colours indicate bacterial phyla. (b) Distribution of functional capability of cultured skin bacterial isolates against *Bsal*. The numbers represent the total number of isolates with the respective function. (c) Bacterial density on salamander skin throughout the first 28 days of the experiment. The arrow indicates time of *Bsal* exposure. Treatments are labelled as follows: control* = daily addition of a sham treatment of 1 ml of sterile distilled water with an agar swab, control = no treatment, pseudo = daily addition of a *Pseudomonas* sp., and steno = daily addition of a *Stenotrophomonas* sp. Asterisks are used to denote time points where significant differences among treatments were detected. (d) Survival probability curves across experimental treatments. The pseudo treatment exhibited significantly greater survival compared to the control. (e) (Inset) Rate of change in *Bsal* infection intensity throughout the first 28 days (zoospores/day). The asterisk denotes significant differences among treatments. Error bars represent standard error of the mean (c and inset e), and shaded regions represent 95% confidence intervals (d).

intensity throughout the first 28 days differed among treatments (KW $\chi^2 = 7.24$, $p = 0.06$; figure 3e). More specifically, when comparing the two bacterial treatments to address the hypothesis that bacteria can differentially affect infection dynamics, there was a significant difference among treatments, with the *Bsal*-inhibitory bacteria group exhibiting a lower rate of increase in infection intensity compared to the *Bsal*-enhancing bacteria group (KW $\chi^2 = 3.92$, $p = 0.047$). There was also evidence for a difference in survival probability among treatments (Cox log-rank test, $\chi^2 = 7.76$, $p = 0.05$; figure 3d); the *Bsal*-inhibitory bacteria group exhibited slower rates of mortality compared to the control treatment (pairwise log rank: $p = 0.018$, all other comparisons $p > 0.1$). One individual within this treatment group also survived the duration of the experiment, and was no longer positive for *Bsal* after 42 days.

4. Discussion

Advances in the understanding of symbiotic microbiomes are changing our perception of animal biology, including the

ecology of disease in host–pathogen systems [3,5,42]. Our study investigated fire salamander skin microbial communities in the context of the emerging pathogen, *Bsal*, finding that microbial interactions can both elicit protection from disease and contribute to disease pathogenesis. While daily addition of *Bsal*-inhibiting bacteria was able to slow disease progression, the markedly low densities of cutaneous bacteria in unmanipulated settings likely limit their protective capacity. Moreover, selected bacteria that became more abundant following *Bsal* infection coincide with bacteria involved in septicemic events, suggesting a contributing role in disease pathogenesis.

Our study suggests that the abundance of bacteria on fire salamander skin is relatively low. There is limited knowledge in the literature on densities of skin microbiota on hosts, even for human skin; however, our estimates for salamander skin are some orders of magnitude lower than the available estimated density on human skin: 5.7×10^3 rRNA copies mm^{-2} versus $< 10^{11}$ mm^{-2} (approximately 10^8 mm^{-2}) [43]. Interestingly, this estimate of bacterial density on

human skin is derived solely from an old culture-based study (1987), which likely underestimates abundances. To date, few novel systematic studies have attempted to thoroughly address cutaneous microbial density. It is striking how scarce current data on cutaneous microbial density is, for human and amphibian systems alike, and clearly this topic warrants thorough study. It is also likely that these densities will differ among amphibian host species, further meriting comparative investigation.

Bacterial abundance on salamander skin may be low as a result of host investment into alternative defence strategies. A host's microbial community can be seen as a trait that is an extension of the host immune system [1,44]. Selective pressure on the host may lead to evolution of a mucosal environment that is particularly suitable for protective symbionts [45]. While microbial defences can be a significant component of a host's defence strategy, at times even replacing host-produced defences, investing in them can be costly for the host [7,46]. In this context, a host may only be able to invest resources in maintaining either defensive microbiota or host-based defences. The extent and nature of the epidermal mucosal layer undoubtedly differ among amphibian species, and likely shape density and potentially spatial distribution of skin microbes. Fire salamanders have a relatively minimal mucus layer on their skin in comparison to, for example, ranid frogs, which may not favour substantial microbial colonization and proliferation on the skin due to low resource availability. Alternatively, fire salamanders may maintain a strict, active control of microbial populations on their skin, investing resources in host-based defences rather than microbial-based defences, and thus limiting the development of a cutaneous environment that is conducive to microbial establishment and persistence.

Bacterial density and their spatial distribution can have strong implications on the function of these microbial residents [47,48]. In fact, microbial abundances rather than taxonomic shifts of particular taxa in the human gut microbiome have been found to be a fundamental driver of disease [49]. Amphibian skin microbiota are known to produce antifungal compounds [50] which can inhibit colonization and growth of fungal pathogens [51]. Many microbial taxa communicate via a form of cell-to-cell communication known as quorum sensing [52]. Such communication relies on signal build up from many bacterial cells and facilitates group behaviours that can lead to inhibition of colonization by other microbes [42,53]. Likewise, antibiotic activity against pathogens can be seen as a by-product of bacterial interference competition [54], and to participate in such competition bacteria most likely need to be in close proximity [48]. If microbial density is key, the low observed density on fire salamander skin could be inadequate to modulate quorum sensing or interference competition. Thus, the natural microbiota of these amphibians may be insufficient in eliciting large-scale effects on *Bsal* infection dynamics. Such a density-dependent effect is further supported by the artificial addition of *Bsal*-inhibitory bacteria to fire salamander skin instigating changes in infection dynamics. On the other hand, we do not fully know the spatial distribution of the bacterial residents; if spatially aggregated within specific skin regions, bacteria could engage in quorum sensing and/or interference competition. Even if the overall density is low, it may still be possible for bacteria to quickly proliferate under certain conditions,

becoming locally abundant on particular skin regions and able to exert an important defensive effect.

Pathogen invasion is known to alter host microbial communities [55,56]. Cutaneous infection by the related amphibian fungus, *Bd*, has been documented to alter the diversity and composition of skin-associated microbiota on anuran hosts [57,58]. Experimental *Bsal* infection of fire salamanders counters this and did not alter total bacterial density, alpha diversity or community structure (i.e. beta diversity) of the skin bacterial communities as a whole. Despite the lack of overall effects on beta diversity, selected bacterial taxa were found to exhibit differential relative abundance between *Bsal*-exposed and control salamanders. In fact, a *Chryseobacterium* sp. found to be differentially abundant on the skin of *Bsal*-exposed individuals matched cultured liver isolates associated with observed septicemic events in *Bsal*-infected salamanders.

Septicemia may be a mechanism of mortality in *Bsal*-induced chytridiomycosis. Deep ulcerations induced by *Bsal* infection [18] result in breaching of the skin barrier, which likely allows bacteria to invade internal organs. The observed septicemic events may be a result of typically commensal skin bacteria becoming opportunistic pathogens and invading internal organs. Most of the bacteria isolated from the liver are not commonly known pathogens. Many were common residents of environmental substrates as well as in host skin and gut microbiomes; however, several of them have previously been documented as opportunistic pathogens, associated with cases of infection and bacteremia in fish, plants and humans (e.g. [59–62]; electronic supplementary material, table S2). Specifically, *Acinetobacter*, the most common bacterium isolated from the liver, has been associated with bloodstream infections (e.g. [42]), and also with skin lesions of another amphibian, the Hellbender [63].

Host-associated microbial communities are also known to affect disease dynamics [4,64–66]. *In vitro*, we found that cultured resident bacteria displayed a range of functional capacities against *Bsal*, including inhibition and enhancement. Such interactions between invading pathogens and host symbionts have been documented across multiple amphibian systems as well as other host systems [26,64,67–70]. In the conducted bacterial addition experiment we were able to, in part, replicate the *in vitro* function of these bacteria *in vivo*. We found that *Bsal*-enhancing bacteria had no effect on salamander survival. In general, *Bsal*-induced chytridiomycosis manifests itself very quickly on fire salamanders [19]. The average time until [death] in controls was 29.5 days, which is only 3–4 generations of *Bsal*; thus, in this hyper-susceptible host it is likely to be rather difficult to hasten the onset of disease, even if *Bsal*-enhancing bacteria are present in high abundance. This fact may explain the observed findings for the *Bsal*-enhancing treatment. On the other hand, the *Bsal*-inhibitory bacteria resulted in a slower build-up of *Bsal* infection intensity in comparison to the *Bsal*-enhancing treatment and a marginal increase in survival compared to the controls. These findings demonstrate that bacteria can differentially affect infection at least under certain conditions, which has also been observed in selected hosts infected with *Bd* (e.g. [47]). The inhibitory bacterium used was a *Pseudomonas* sp.; pseudomonads are known in multiple systems to be antifungal and have disease-suppressing properties [71–73]. Importantly, within the conducted experiment investigating *in vivo* function, skin bacterial densities

on salamanders in bacterial addition groups were greater than those found to occur naturally on salamanders in this study; thus, density may be key for bacteria to elicit a protective function [47,49]. It is also worth highlighting that there was one individual within the *Bsal*-inhibitory bacteria group that survived the duration of the experiment (77 days), which has never been observed in untreated fire salamanders so far, neither in laboratory trials, nor in the wild.

The conducted experiment demonstrates that bacteria can shift infection dynamics within certain frameworks. The potential of bacteria to affect *Bsal* infection dynamics leaves the door to exploring probiotics open. In the sole survivor, *Bsal* infection was no longer present after 42 days, suggesting that clearance had occurred; such a prolonged course of infection, however, also involves prolonged pathogen shedding, which has been shown to be highly unfavourable to overall outcomes of disease outbreaks [74]. Any future work to develop probiotics, single species or probiotic mixtures, should focus on bacteria (or consortia) that prevent pathogen colonization or result in more rapid pathogen clearance. Such probiotics could cull disease progression and minimize transmission. The feasibility of achieving this on a large scale and thereby shifting disease dynamics at biologically meaningful levels, however, will certainly be a challenge.

Are skin bacteria on fire salamanders friends, foes or bystanders? Our data suggest that bacteria living on fire salamander skin may simply be *bystanders*, unable to provide sufficient protection against *Bsal*, perhaps due to low bacterial numbers combined with *Bsal*'s ability to disseminate inside salamander skin, thus averting bacterial defences. Furthermore, disease-induced septicemia by potentially opportunistic pathogens within the skin microbiota presents selected

bacteria as a clear *foe* to fire salamanders. However, dampening of *Bsal* infection by the addition of *Bsal* inhibitory bacteria suggests that skin bacteria can be a *friend* to the salamander under certain conditions. The multifaceted nature of host microbiota highlights the complex relationship of hosts, their microbiomes and disease, and underscores the importance for continued research in this field.

Ethics. All work with animals has been approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2015/ 83 and EC 2016/73).

Data accessibility. All OTU tables, infection data and metadata are available in the Dryad Digital Repository at: <http://dx.doi.org/10.5061/dryad.m40t6g7> [74]. Illumina sequence data are available on SRA under Bioproject PRJNA477390 (SRA accession SRP151078). Bacterial isolate sequences have been archived on GenBank (MH512108–MH512801 & MH523105–MH523149).

Authors' contributions. M.C.B., M.V., S.S., F.P. and A.M. designed research; M.C.B., A.M., M.K., J.S.P., E.B., S.V.P., F.B. and W.B. performed research; M.C.B., A.M. and M.K. analysed data. All authors contributed to writing and revising the paper.

Competing interests. The authors declare having no competing financial interests.

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