Supplementary Material

Disruption of skin microbiota contributes to salamander disease

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Supplementary Methods

Bsal zoospore harvesting for inhibition assays:
Bsal zoospores were obtained from filtering *Bsal* cultures maintained in culture flasks. More specifically, after 5 days of growth in TGHL media this media was exchanged with sterile artificial pond water and incubated overnight. This stimulates the release of zoospores. The next day, the water was collected from the flask and centrifuged to pellet the released zoospores. The pelleted cells were then re-suspended in fresh media and filtered to remove any sporangia. The filtered zoospore suspension was then quantified, diluted to $1x10^6$ zoospores/ml, and added the assays wells.

Detailed methods for *Bsal* growth inhibition assays Briefly, zoospores were obtained from filtering *Bsal* cultures maintained in culture flasks (see supplemental methods for detailed protocol). *Bsal* zoospores were grown in the presence of the cell-free supernatant of each bacterial isolate in triplicate. Assay plates were incubated at 18° C for 7 days and optical density readings occurred on day 0, 4, 7 and 10. Bacterial CFS was collected from liquid cultures grown for 3 days on a shaker (250 rpm) and filtered through 0.22 μm filter. Bacteria were not co-cultured with *Bsal* during cell-free supernatant preparation (CFS).

Bacterial selection criteria for bacterial addition experiment: Bacteria were selected using the following criteria: (1) consistent and distinct function across bacterial isolates assigned to the same bacterial OTU at 97% similarity (either consistent inhibition or enhancement in > 50% of cultured isolates), (2) commonly cultured from skin (cultured from > 5 individuals), and (3) present in fire salamander skin communities using next generation sequencing approaches.

Detailed methods for 16S rRNA characterization of communities: Only forward reads were used because reverse reads typically suffer from lower quality [35]. Quality filtered sequences were clustered into sub-operational taxonomic units (sOTUs) using Deblur [36]. Within this workflow, sequences were trimmed to 150 bp to maintain only high quality bases within each sequence, and sOTU clusters with less than 10 reads were removed as per the recommendations in Bokulich et al. [37]. Taxonomy was assigned with the naïve Bayesian Classifier and the RDP database [29]. A phylogenetic tree was built in QIIME using fasttree [30]. Samples were subsequently normalized at 5,818 reads per sample (depth chosen based on read count of lowest sample). The alpha diversity measures, OTU richness, Effective number of sOTUs (exp(Shannon)) [38], and Faith's phylogenetic diversity were calculated for all samples, and beta diversity (i.e. community structure) was calculated as the weighted Unifrac, unweighted Unifrac, Bray Curtis and Binary Jaccard pairwise distances in QIIME.

Detailed methods for MALDi-TOF: The samples were cultured for 24 hours at 15°C on Columbia agar with sheep blood (Oxoid, Wesel, Germany). One colony per sample was smeared upon a MALDI polished steel target plate, covered with 1 μl HCCA matrix and, after air drying, loaded into the MALDI-TOF mass spectrometer. The spectra were obtained in linear positive mode with set-up values set as follows: ion source 1 voltage, 19,5 kV; ion source 2 voltage, 18,3 kV; lens voltage, 7 kV; mass range, 2–20 kDa; peak resolution, >400. The final spectrum was the sum of 6 single spectra, each obtained by 200 laser shots on random target spot positions (1200

shots in total). Spectra acquisition, peak picking, baseline subtraction, smoothing and final identification were performed with the standard biotyper settings of the MBT Compass software version 4.1. (Bruker Daltonik), which included a RUO database of 6,120 mean spectra projections (MSP) supplemented with the MSP's of the respective *Stenotrophomonas* and *Pseudomonas* isolates, obtained according to the manufacturer's guidelines. Identifications of the respective isolates was reported for score values higher than 2.

Supplementary Tables and Figures

Supplementary Table 1. Field sampling locations across Germany and sample sizes for skin microbiome analysis. Locations with asterisk were also used for qPCR estimates of bacterial abundance.

	Locations	# individuals
Eifel*	ER	2
	Fischbach*	38
	Haftenbach	40
	Kallerbach*	18
	Lamersiefen	18
	Rosbach	46
	Sauerbach	22
	Solchbachtal*	14
	Weisse Wehe	7
	Zweifallshammer	33
Harz		8
Solling		29

Supplementary Table 2. Sample sizes and the number of bacterial isolates cultured from the skin of fire salamanders from multiple populations across Germany.

Location	# bacteria isolates	# individuals
Eifel	83	6
Harz	47	7
Kottenforst	176	34
Liekwegen	25	2
Solling	140	21
Waldeck	161	15
Wolfsburg	76	9

Supplementary Table 3. Functional capacities of bacterial isolates isolated from fire salamander skin that were re-tested multiple times using optical density-based growth assays. **I = inhibiting, E = enhancing, N= no effect on growth**

	Growth assay replicates				
Bacterial isolate ID	**1**	**2**	**3**	**4**	**5**
205	I	E	N		
335	I	E	N		
281	I	E	I		
473	I	E	E	E	E
798	I	E	E	E	E
737	I	E	E	E	E
908	E	N	N	I	N
849	E	I	I	I	I
850	E	I	I	I	I
235	E	N	I		
277	E	N	I		
540	E	N	I		
393	N	N	I		
23	N	N	N	E	E
2	N	E	N		
369	N	N	N	N	E
370	N	E	N		

Supplementary Table 4. Differentially abundant bacterial taxa between *Bsal* exposed and control fire salamanders as identified through the LEfSe method. Bacterial taxa with an asterisk indicate which ones are presented in Figure 1.

Treatment	Bacterial Taxa	LDA	p-value
Control	Actinobacteria Actinobacteria Actinomycetales	4.560	0.006
	Actinobacteria Actinobacteria Actinomycetales	4.560	0.006
	Actinobacteria Actinobacteria Actinomycetales OTU0048*	4.560	0.006
	Actinobacteria Actinobacteria Actinomycetales Microbacteriaceae *Leucobacter* OTU0124*	3.562	0.030
	Bacteroidetes Cytophagia	3.829	0.016
	Bacteroidetes Cytophagia Cytophagales	3.829	0.016
	Bacteroidetes Cytophagia Cytophagales Cytophagaceae	3.829	0.016
	Bacteroidetes Cytophagia Cytophagales Cytophagaceae *Dyadobacter*	3.726	0.037
	Bacteroidetes Cytophagia Cytophagales Cytophagaceae *Dyadobacter* OTU0064*	3.612	0.037
	Bacteroidetes Sphingobacteriia Sphingobacteriales Sphingobacteriaceae *Pedobacter*	3.673	0.025
Infected	Bacteroidetes Flavobacteriia Flavobacteriales Weeksellaceae *Chryseobacterium* OTU0231*	3.594	0.022
	Firmicutes Bacilli Lactobacillales	3.545	0.036
	Firmicutes Bacilli Lactobacillales Streptococcaceae *Lactococcus* OTU0204*	3.585	0.026
	Fusobacteria	3.623	0.025
	Fusobacteria Fusobacteriia	3.623	0.025
	Fusobacteria Fusobacteriia Fusobacteriales	3.623	0.025
	Fusobacteria Fusobacteriia Fusobacteriales Fusobacteriaceae	3.623	0.025
	Fusobacteria Fusobacteriia Fusobacteriales Fusobacteriaceae	3.634	0.025
	Fusobacteria Fusobacteriia Fusobacteriales Fusobacteriaceae OTU0029*	3.634	0.025
	Proteobacteria Gammaproteobacteria Aeromonadales	4.118	0.036
	Proteobacteria Gammaproteobacteria Aeromonadales Aeromonadaceae	4.118	0.036
	Proteobacteria Gammaproteobacteria Aeromonadales Aeromonadaceae	4.118	0.036
	Proteobacteria Gammaproteobacteria Xanthomonadales Xanthomonadaceae *Stenotrophomonas acidaminiphila* OTU0014*	3.594	0.006

Supplementary Table 5. Taxonomic identity and quantity of bacterial isolates cultured from liver tissue of *Bsal*-infected fire salamanders. Common habitats, host and associations based on NCBI Blast hits are provided with pathogen and disease related associations in bolded text.

Taxonomic identity	# isolates	# salamanders	Common habitats, host and associations
Acinetobacter johnsonii (Proteobacteria)	15	8	amphibian skin associated microbiome (Madagascar); plant endophyte; **cerebrospinal fluid in humans with meningitis**, wastewater; water environments; microbiota of marine sponges; **emerging fish pathogen**
Chryseobacterium sp. (Bacteroidetes)	9	5	amphibian skin-assoiciated microbiota (Madagascar, New Zealand); soil bacterial communities; wastewater effluent; fish-associated microbiome; dairy environment, **disease aquatic animals**; plant-associated phyllosphere; human skin microbiome; **diseased skin microbiome**
Sphingobacterium faecium (Bacteroidetes)	3	3	truffle-associated bacteria; geothermal soils; aquatic rock associated biofilms; soil and water associated microbiomes; amphibian skin-associated microbiome; **bacteria on disease Brassicaceae plants**; fish gut-associated microbiome;
Sphingobacterium multivorum (Bacteroidetes)	3	1	endophytic plant bacteria; lichen-associated bacteria; soil environments; plant rhizosphere
Flavobacterium succinicans (Bacteroidetes)	2	1	aquatic rock assoiciated biofilms; cockroach microbiota; fish-associated microbiome; soil environments; plant-associated rhizosphere; **diseased fish**
Myroides sp. (Bacteroidetes)	2	1	phototrophic river biofilms; amphibian skin associated microbiomes (Madagascar); Bat guano; *Drosophila* microbiota; environmental microbiota; plant-associated phyllosphere; swine lagoon; river biofilms; soil rhizosphere
Erwinia sp. (Proteobacteria)	2	2	bacterial communities associated with aphids; bark beetle microbiota; bacteria on fresh fruit and vegetables; raw milk; mosquito-associated bacteria
Microbacterium maritypicum (Actinobacteria)	2	2	coastal environments; bird plumage microbiota; soil environments; amphibian skin-associated microbiomes

Arthrobacter sp. (Actinobacteria)	1	1	microbiota of cave environments, microbiota of marine sponges; marine and estuarine habitats; bacterial endophyte of plants; isolate from the surface of cheese; human skin microbiome;
Arthrobacter psychrolactophilus (Actinobacteria)	1	1	alpine soil and glacier cryoconite, Antarctic environments, soil from a bamboo plantation, sewage
Flavobacterium sp (Bacteroidetes)	1	1	**skin microbiome associated with disease flares and treatment in children with atopic dermatitis**; soil environments; plant-associated phyllosphere
Enterobacteriaceae sp (Proteobacteria)	1	1	plant roots; nemotode gut microbiota; soil environments; **bacteria on disease Brassicaceae plants**; beetle-associated microbiota
Erwinia dispersa (Proteobacteria)	1	1	bacterial communities in boreal forest mushrooms; isolates from purple siltstone; gut symbionts in stinkbugs; plant root-associated bacteria
Klebsiella sp. (Proteobacteria)	1	1	plant-associated microbiota; soil environments; water environments; nemotode associated microbiota; date palm rhizosphere
Acinetobacter guillouiae (Proteobacteria)	1	1	amphibian skin associated microbiota (captive Atelopus); nemotode associated microbiota; river sediment; **bacteria on diseased Brassicaceae plants**; plant rhizosphere
Unclassified bacteria	1	1	closest match 95% to Aeromonas salomicida - an etiological agent for furunculosis, **a disease that causes septicemia, haemorrhages, muscle lesions, inflammation of the lower intestine, spleen enlargement, and death in freshwater fish populations.**

Supplementary Table 6. Re-isolation of bacterial isolates added to fire salamander skin throughout the experiment. Yes indicates positive detection of added bacterium, No indicates negative detection, and '--' indicates time points were individuals were no longer in the experiment.

Treatment	Individual ID	7d	14d	21d	28d	35d	42d	49d	56d	63d	70d	77d
Pseudomonas sp.	**8**	No	No	Yes	No	--	--	--	--	--	--	--
	11	No	Yes	Yes	Yes	No	--	--	--	--	--	--
	12	Yes	No	Yes	No	No	No	No	No	No	--	--
	16	No	No	No	No	--	--	--	--	--	--	--
	18	Yes	No	No	No	Yes	--	--	--	--	--	--
	19	No	Yes	Yes	No	--	--	--	--	--	--	--
	27	No	Yes	No	No	No	No	No	Yes	No	No	No
Stenotrophomonas sp.	**1**	No	No	No	No	Yes	No	No	--	--	--	--
	2	Yes	No	No	Yes	--	--	--	--	--	--	--
	3	No	No	No	No	--	--	--	--	--	--	--
	4	Yes	No	Yes	No	--	--	--	--	--	--	--
	7	Yes	No	No	Yes	--	--	--	--	--	--	--
	15	No	No	Yes	Yes	No	--	--	--	--	--	--
	24	No	Yes	Yes	No	--	--	--	--	--	--	--

Supplementary Figure 1. Bacterial taxonomic composition of fire salamander in natural populations at the order level. Ten populations from the Eifel region in Western Germany are including along with two populations (Harz and Solling) from Central Germany.

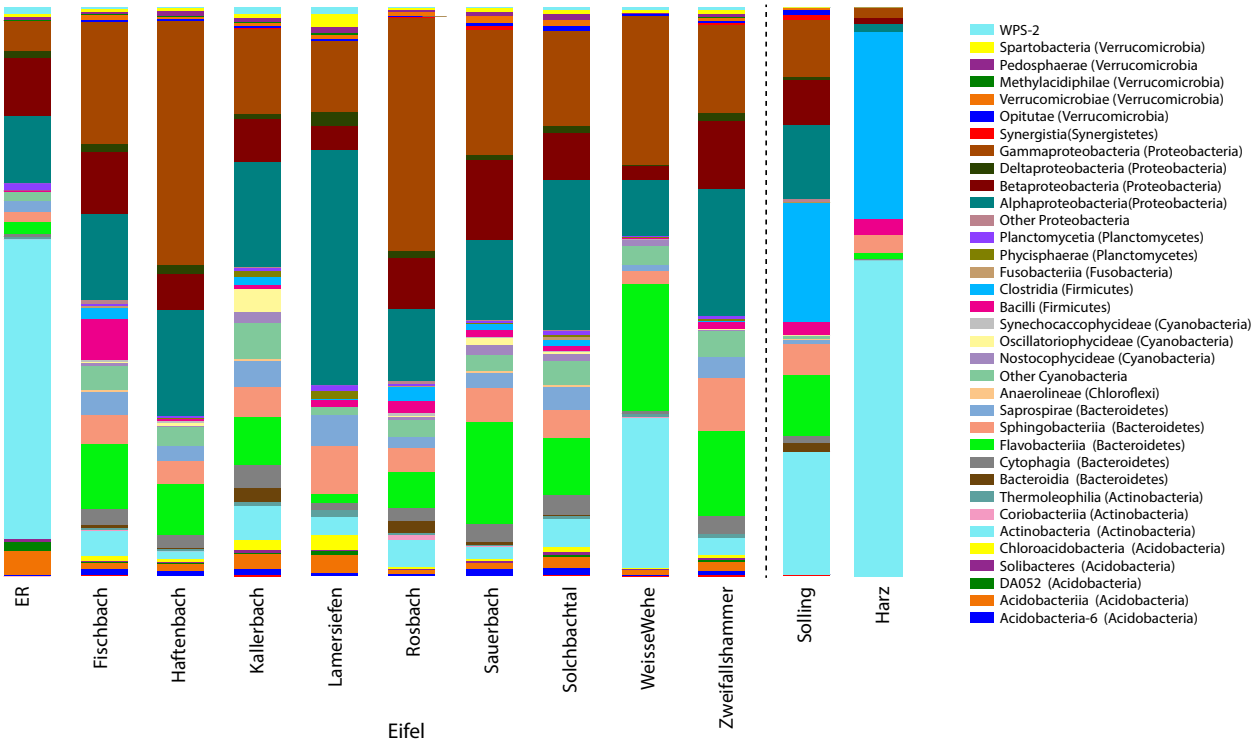
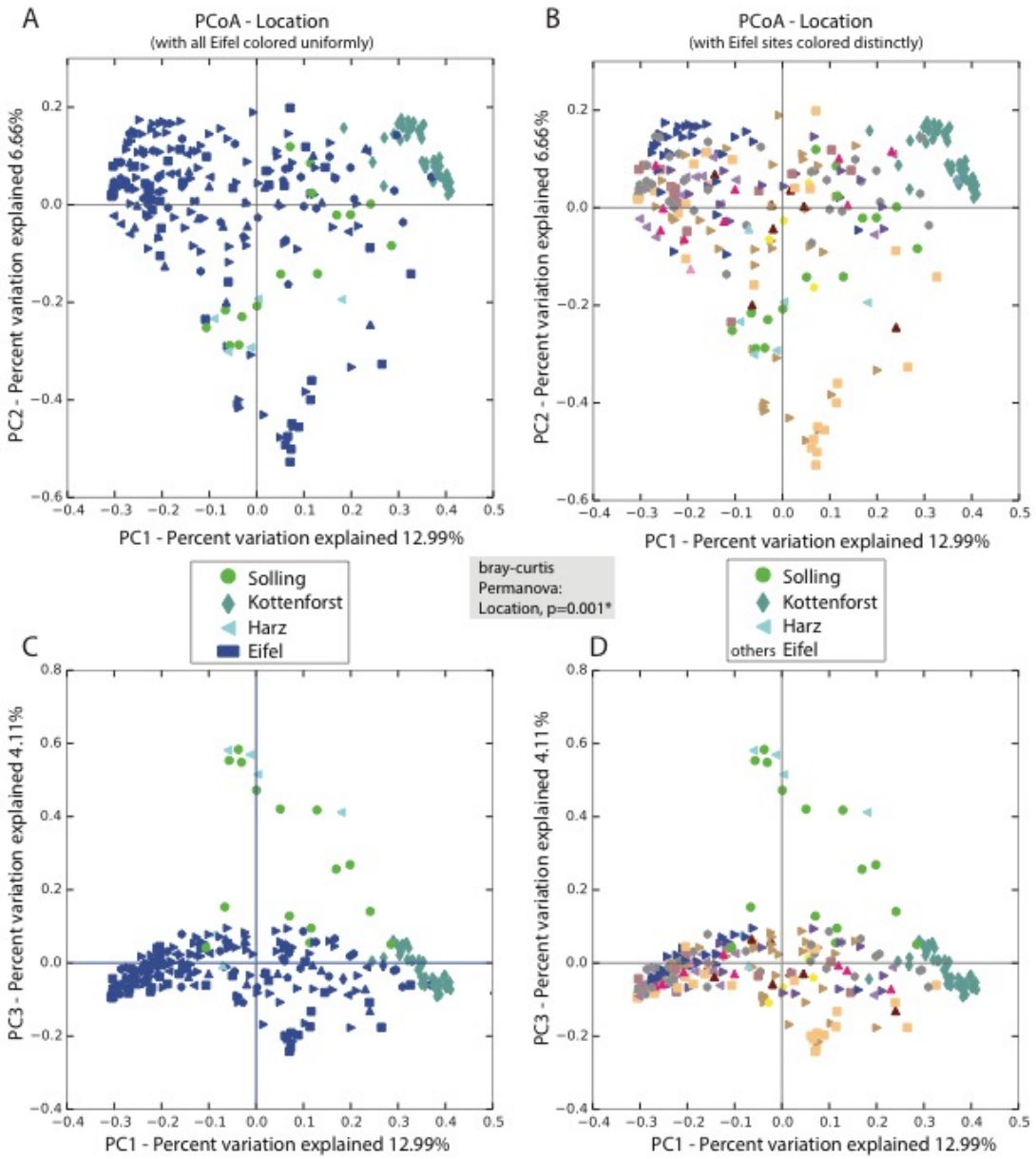


Figure S2. Principle Coordinate Analysis of Bray-Curtis distance of fire salamander skin microbial communities from multiple regions and locations across Germany. Skin microbial community structure differed significantly across locations. (A&B) display sample separation on PCo axis 1 and 2. (C&D) display sample separation on PCo axis 1 and 3. Plots (B) and (C) differentiate sites from the Eifel region of Germany.



Supplementary Figure 3. Alpha diversity of salamander skin microbiota in response to *Bsal* infection. Blue color indicates the before exposure time point and orange indicates the after time point (i.e. 10 days post infection). Statistical results from linear mixed models are provided evaluating the factors of time (Before vs After) and treatment (Bsal vs Control).

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