**Description and comparison of the skin and ear canal microbiota of non-allergic and allergic German shepherd dogs using next generation sequencing**

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Short title*:* Skin and ear canal microbiota in non-allergic and allergic German shepherd dogs

Abstract

Atopic dermatitis is one of the most common skin diseases in dogs. Pathogenesis is complex and incompletely understood. Skin colonizing bacteria likely play an important role in the severity of this disease. Studying the canine skin microbiota using traditional microbiological methods has many limitations which can be overcome by molecular procedures. The aim of this study was to describe the bacterial microbiota of the skin and ear canals of healthy non-allergic and allergic German shepherd dogs (GSDs) without acute flare or concurrent skin infection and to compare both. Bacterial 16S rRNA gene amplicon sequence data revealed no differences of bacterial community pattern between the different body sites in non-allergic dogs including axilla, front dorsal interdigital skin, groin and ear canals. The ear canal microbiota of non-allergic GSDs had a reduced diversity compared to their skin microbiota. The bacterial community composition of the skin and ear canals of allergic dogs showed body site specific differences compared to non-allergic dogs. The bacterial microbiota of allergic dogs were characterized by lower species richness compared to non-allergic dogs, only for the samples obtained from the axilla. Actinobacteria was the most abundant phylum identified from the non-allergic dogs andProteobacteria from allergic dogs. *Macrococcus* spp. were more abundant on non-allergic skin while *Sphingomonas* spp. were more abundant on the allergic skin.

**Keywords:** canine; skin; ear canal; microbiome; microbiota; sequencing; 16S rRNA; atopic dermatitis; Macrococcus; Staphylococcus; dysbiosis; German shepherd dogs; veterinary; dermatology; cutaneous; microbiology; inflammatory; prutitus; abundance; species; ecosystem; NMDS; diversity; oclacitinib

Introduction

Several next generation sequencing (NGS) studies in the last few years have shown that the skin of dogs, similar to humans, contains a higher diversity of bacterial taxa than previously believed [1–4]. Bacteria play an important role in both health and disease and changes in bacterial community composition of the skin are associated with many skin diseases in both humans and animals [2,5].

Canine atopic dermatitis (cAD) is a common skin disease in dogs characterized by a genetically predisposed inflammatory, IgE-associated, pruritic allergic disease, affecting certain body sites and ear canals [6,7]. CAD has been proposed as an animal model for human atopic dermatitis [8,9]. Environmental allergens are the most common cause of cAD [6]. But, food allergens (cutaneous adverse food reactions; CAFRs) can also cause identical clinical signs or be a flare factor of a cAD, making a clinical differentiation impossible [10,11]. The final diagnosis must be obtained through a systematic workup [12]. Studies in dogs have shown an association between the skin microbiota and allergic skin diseases. The skin of six allergic dogs without signs of pyoderma or *Malassezia* dermatitis revealed that there was a lower bacterial community diversity compared to the skin of 12 healthy dogs, but the bacterial community composition did not differ significantly [13]. Recently, a longitudinal study showed reduced diversity and different bacterial community composition in dogs with cAD and secondary pyoderma compared with healthy dogs [14]. After antimicrobial therapy and remission of skin lesions, the diversity was restored and the clustering difference of the bacterial communities was reduced [14]. Neither study examined the ear canal, which is a commonly affected body site in allergic dogs. The only study using NGS for evaluating the microbiota of asymptomatic ear canals of dogs with cAD showed no difference in the diversity but a significant difference in bacteria community composition [1]. All previously mentioned studies involved dogs from various breeds. It is well documented that cAD has breed predispositions and that the phenotype of the disease differs between breeds [15]. It is unclear if the different phenotypes of cAD affect the bacterial community composition between the breeds. To date no study has evaluated the microbiota of the skin and ear canal of only a single breed in both healthy and allergic skin disease states, thus minimizing potential bias due to allergy phenotype effect on the microbiota. The German shepherd dog (GSD) was chosen in our investigation because it is a high-risk breed for cAD [15], possibly due to an altered expression of the plakophilin 2 gene and other genes of the chromosome 27 [16].

The goal of the study was to describe and compare bacterial microbiota of four body sites (axilla, front dorsal interdigital skin, groin and ear canal) of healthy (non-allergic) and allergic GSDs using a 16S rRNA gene amplicon based Illumina sequencing approach. We hypothesized that atopic dermatitis and/or CAFR influence the microbiota of the skin and ear canal of GSDs resulting in reduced bacterial diversity and significantly different bacterial community composition.

Material and methods

Study subjects

The clinical study was performed at the small animal clinic of the Justus-Liebig University (JLU), Giessen, Germany. Samples were collected at the small animal clinic (JLU) during routine diagnostic appointments specifically for this study. All owners were informed of the procedures and signed a consent form for sample collection. The Animal Welfare Committee of the Justus-Liebig University of Giessen was informed about the study protocol and especially the sampling method was discussed. As there is neither pain, harm nor damage caused by gently rolling a cotton swab on skin, they assured us that ethical approval by the responsible authority is not required. Two groups of GSDs were studied. The control group included 12 GSDs without any history of allergic conditions or any clinical skin/ear canal lesions compatible with allergy (they will be referred to as "non-allergic" in the rest of the manuscript) at the time of examination and sampling. In order to minimize the risk of including a dog with subclinical allergy, only dogs older than four years old were involved, as allergic conditions most often develop in young dogs from 6 months to 3 years [17]. In order to investigate a possible influence of the household conditions to the microbiota, two non-allergic GSDs per household were included. Neither systemic antibiotics nor any immunomodulatory or anti-inflammatory drugs were allowed six months prior to sampling. Bathing with shampoo and the use of ear cleaners was not allowed seven days prior to sampling. Twelve adult allergic GSDs were diagnosed with cAD, either due to cutaneous food reactions or environmental allergens. Standard diagnostic and therapeutic methods were used, including fullfillment of at least five of Favrot´s criteria and excluding other pruritic dermatosis (e.g. flea saliva hypersensitivity, sarcoptic mange) [12,18]. A combination of cAD and/or CAFRs with flea saliva hypersensitivity was allowed. Dogs with any secondary bacterial or fungal skin or ear infection were excluded. Systemic and topical antibacterial or antifungal agents were not allowed 30 and 14 days prior to sampling, respectively. Systemic administration of any immunomodulatory or anti-inflammatory drugs, with the exception of oclacitinib, were equally not allowed two months prior to sampling. Topical immunomodulatory or anti-inflammatory drugs had to be withdrawn 14 days prior to the study. Any shampooing and ear cleaners were not allowed seven days prior to sampling. All owners filled out a questionnaire regarding their dog’s housing, partner animals, current diseases and treatment, food supplements, frequency of bathing and type of shampoo. Owners of allergic dogs were asked about gastrointestinal signs and to score their dog’s pruritic signs using a pruritus visual analogue scale (pVAS), as previously validated [19,20].

Assessment of lesions

All dogs were examined clinically and dermatologically including otoscopic examination using sterile powder-free gloves and an autoclavable metal speculum and cytology. A validated site-specific lesion and scoring scale of lesions‘ severity, the Canine Atopic Dermatitis Extent and Severity Index (CADESI-4), was performed for the atopic dogs in order to evaluate any relationship of cutaneous microbiota and the severity of atopic dermatitis [21]. Skin and ear canal cytology was performed as described [22,23].

Sample collection

Prior to sampling and in between sampling of the GSDs, the examination table was cleaned with PCR Clean™ Wipes (Minerva Biolabs, Berlin, Germany) according to the manufacturer manual, to avoid DNA cross-contamination. In order to minimize microbial contamination from the clinic´s floor, dogs were brought directly into the examination room and placed onto the examination table. After physical examination, the left axilla (A), left front dorsal interdigital region (Int), left side of the groin (L) and the left ear canal (O) were sampled. These body sites appear to be most commonly affected by atopy in this breed [15]. For sampling we used 70% ethylene oxide sterilized forensic swabs with transport tube, polystyrene stem material and viscose swab material (Forensic Swab, Nr 80.629, Sarstedt, Nuembrecht, Germany) to ensure the absence of DNA contamination and to avoid cotton or wood mitochondria from the swab or the stem. The swabs were rubbed 40 times on the desired region, rotating one-quarter of the swab´s site (90°) for 10 times. All samples were obtained in duplicates. Subsequently, a sample was taken for cytological purposes using a sterile cotton swab. The samples were transported immediately at 8°C to the Institute of Applied Microbiology, JLU Giessen, for storage and further processing. Samples were stored at -20°C until all samples were collected.

DNA extraction and 16S rRNA gene amplicon sequencing

Total DNA was extracted from DNA free swabs using the NucleoSpin® 96 Soil kit (96-well extraction system, Macherey Nagel AG, Oesingen, Switzerland) which can efficiently extract DNA from Gram-negative and Gram-positive bacteria including spores. According to manufacturer’s instruction using vacuum processing (NucleoVac 96 vacuum manifold, Macherey Nagel AG, Oesingen, Switzerland) with slight modifications, extraction was started using lysis buffer SL1 and afterward steps 1 to 5 were repeated with lysis buffer SL2, thus samples were lysed twice. Total DNA was eluted with approximately 80 µL PCR water (1x 30 µL, 1x 50 µL) instead of SE buffer. DNA was quantified spectrophotometrically using a NanoDrop spectrophotometer (Thermo Scientific) and subsequently checked for the presence and amplifiable 16S rRNA gene sequences for selected samples.

The 16S rRNA gene sequences of *Bacteria* were amplified for Illumina amplicon sequencing (LGC Genomics, Berlin, Germany) using a nested PCR approach with a first PCR with the primer system - 341F/1061R (V3-V6) (20 cycles) followed by a second PCR with primer system 515F-Y and 926R-jed (V4-V5) (20 cycles), because according to previous studies, only a low amount of microbial DNA was detected on human skin analyzed by PCR [24,25]. For each sample, forward and reverse primers in the nested PCR had the same 10-nt barcode sequence. The first round of PCR was carried out for 20 cycles, using the following parameters: 2 minutes 96°C pre-denaturation; 96°C for 15 seconds (s), 50°C for 30 s, 70°C for 90 s and primers without inline barcodes were used (341F/1061R). For the second round, 1 μl PCR product from the first round was used and the PCR conditions were the same as before. In this case, barcoded primers were added (515F-Y/926R-jed). DNA concentration of amplicons of interest was determined by gel electrophoresis. About 20 ng amplicon DNA of each sample were pooled for up to 48 samples carrying different barcodes. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on MiniElute columns (Qiagen). About 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled, and size selected by preparative gel electrophoresis. Sequencing was done on Illumina MiSeq using V3 Chemistry (Illumina). Raw sequence reads are available in the Sequence Read Archive (SRA) with BioSample Accession numbers SAMN14565128 to SAMN14565223 in the BioProject PRJNA624030.

**Amplicon sequence data analysis**

The NGS analysis pipeline (https://www.arb-silva.de/ngs) of the SILVA rRNA gene database (SILVAngs 1.3) was used for sequence analysis [26]. For this, datasets of all combined sequence reads were uploaded to the database. All reads were aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision21008)) [26], against the SILVA SSU rRNA SEED and quality controlled [27]. Reads shorter than 50 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, were excluded from further processing. Reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA) as well as putative contaminations and artifacts, were identified and excluded from downstream analysis. The next process step was dereplication and clustering with cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) using *accurate mode*, ignoring overhangs and applying identity criteria of 1.00 and 0.98 respectively [28]. For classification a local nucleotide BLAST search was performed against the non-redundant version of the SILVA SSU Ref dataset (release 128; http://www.arb-silva.de) using blastn (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings [29]. Unique reads were clustered in operational taxonomic unit (OTU) on a per sample basis under the criterium of 98% sequence identity to each other (pairwise distance and single linkage clustering). The longest read in each cluster was classified as the reference for each OTU and was mapped onto all reads that were assigned to the respective OTU. OTUs were asigned to taxonomic paths (resolved at the genus level). Several OTUs were thereby partially asigned to the same taxonomic path /genus). This process resulted in quantitative information (number of individual reads of all OTUs per taxonomic path), despite the PCR limitations, possible sequencing technique biases and multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the function “(% sequence identity + % alignment coverage)/2” did not exceed the value of 93, remained unclassified and were assigned in the virtual taxonomical group “No Relative” in the SILVAngs fingerprint and Krona charts [30], as previously reported [31,32].

Additionally, the datasets were normalized by rarefying randomly to 7,500 reads per sample. Three samples (a17O, a17INT and a16O) had sequence reads less than 7,500 and were discarded. Diversity analysis was performed for the rarefied dataset too, as above described. The results did not differ from the results based on the unrarefied dataset. Taken into consideration that there is a disagreement about the use of this commonly normalization procedure, among several authors [33], and because the results were not different from the unrarefied dataset, in the manuscript is presented the analysis of the unrarefied dataset. Yet, the analysis of the rarefied dataset is provided in supporting information (S0).

Bacterial communiy anaylsis was performed in PAST3 (https://folk.uio.no/ohammer/past) [34] at the level of phylogenetic groups, which was given by a resolution at the genus level. Alpha diversity was measured by calculating different diversity indices considering the number of phylogenetic groups per samples and the number of reads per phylogenetic group. The Shannon index (overall α-diversity), the Chao 1 index (richness; number of taxa corrected by the presence of singleton), Dominance (=1-Simpson index) and Evenness (Buzas and Gibson´s evenness: eH/S) [35] (both together used to see if individual taxa are dominating the microbiomes or if all taxa are equally distributed) were determined. Rarefaction curve analyses considering the number of phylogenetic groups and the number of reads per phylogenetic group to evaluate richness in different sample sizes [36]. Significant differences between the alpha diversity indices determined for allergic vs nonallergic dogs were further evaluated. For two groups/communities, i) body skin (axilla, interdigital, groin) vs ear canal or ii) body sites between non-allergic versus and allergic dogs, unpaired t-tests were performed in SigmaPlot 13 (Systat Software Inc.). First, a normality test (Shapiro-Wilk) was performed [37]. If the data passed the normality test, a two-tailed p-value was obtained from the t-test analysis. If the normality test failed, the Mann-Whitney Rank Sum Test was applied to test for the presence of significant differences. For more than two groups (all body sites), the significant differences of the alpha diversity values were evaluated by performing a Kruskal Wallis Test [38]. Thebeta diversity (comparison of thephylogenetic composition of the bacterial communities of the different samples) was studied based on relative abundance data analyzed by non-metric multidimensional scaling (NMDS; [39]) using Bray-Curtis similarity matrix [40]. Environmental variables (e.g. body sites, home (living in the same house) and sex) were included to the NMDS plot displayed as biplot vector. One Way ANOSIMs (9999 permutations) [41] and One way PERMANOVA (9999 permutations) [42] were performed to determine significant differences (evaluating the sequential Bonferroni p-values) among samples. SIMPER (Similarity Percentage) analysis [41] also based on the Bray Curtis similarity matrix, was used to determine the average percent contribution of the different taxa to the dissimilarity among samples. Significant differences between the mean relative abundances of specific taxa were evaluated performing a normality test (Shaphiro-Wilk), t-test, Mann-Whitney Rank Sum test or a Kruskal Wallis Test as indicated.

Results

Study subjects

Overall 29 GSDs were sampled but five were excluded according to the exclusion criteria (Table 1 in S1). Twelve non-allergic (6 male, 1 male castrated, 4 intact female and 1 spayed female) and twelve allergic (2 intact male, 1 male castrated and 9 intact female) GSDs were further analyzed (Table 1 in S1). The dogs were living in a radius of max 131 kilometers away from the town of Giessen (Germany). The age of the non-allergic dogs (mean: 7.5 ±1.9 years) was significantly higher (t-Test p< 0.01) than the age of allergic GSDs (mean: 4.8 ±2.2 years). All allergic dogs had mild lesions with a median CADESI-04 score of six (range 0-11) and a median PVAS score of four (range 0-8) (Table 2 in S1). Two dogs had a PVAS score of 7/10 and 8/10, respectively with only mild skin lesions. Six allergic GSDs received labeled doses of oclacitinib (Apoquel, Zoetis Deutschland GmbH, Berlin, Germany).

Analysis of the phylogenetic composition of the GSDs microbiota

In total, 4,038,850 paired end sequences with an average sequence length of 373 nucleotides (nt) were obtained. Briefly, 2,579 sequences (0.06%) were rejected because they failed the SILVAngs pipeline quality control. Finally, 4,036,271 sequences (2334-246525 per sample) were further analyzed (Table 3 in S1). In total 241,114 unique reads (5.97% of the finally analyzed sequences) were assigned to different OTUs. Additionally, 1,129,221 (27.96%) sequences (number of sequence reads with 98% sequence identity to each other; defined as "clustered"), and 2,665,936 (66.01%) sequences (number of sequence reads with 100% identity to another; defined as "replicates") were assigned to OTUs. Each OTU was classified in the SILVA database with a taxonomic paths with maximum resolution at the genus level (phylogenetic groups for unnamed genera). Chloroplast, mitochondrial and archaeal 16S rRNA gene sequences were detected with a relative abundance of 20.13% (190,605 sequences), 0.07% (9,430 sequences), 0.02% (1,064 sequences) of the total analysed sequences, respectively (Table 3 in S1). Sequences that did not match any known taxa (sequence similarity <93% to the next known taxon) were classified as “no relative” and had 0.3% relative abundance (5,131 sequences) (Table 3 in S1). The sequences belonging to the *Archaea*, chloroplasts, mitochondria, and no relative groups were excluded from further analysis. Only sequences assigned to the domain *Bacteria* (3,830,041 in total; 2,334 to 232,445 per sample) were further analyzed and were set to 100%.

Skin Microbiome of non-allergic German shepherd dogs

Alpha diversity of bacterial communities present at skin samples of non-allergic GSDs

Richness (Chao1) and diversity (Shannon) of phylogenetic groups at the four sites (axilla, interdigital, groin, ear canal) were not significantly different between the body sites (Kruskal-Wallis for Shannon index, p= 0.6214; for Chao 1, p= 0.4784).

However, a greater number of individual samples from the interdigital region and ear canal had a more homogeneous composition of microbiota, showing a lower richness (Chao 1 values; Fig 1 in S2). The three skin sites (axilla, interdigital, groin) were evaluated together comparing body skin to ear canal. Shannon values (t-Test, p= 0.889) were similar, but the ear canal showed significant lower bacterial richness (lower Chao-1 values, Figs 1a and 1b) than the body skin sites (t-Test, p= 0.000492; Table 4 in S1) which were more evenly distributed (significantly lower evenness values; t-Test, p= 0.000742; Table 4 in S1, Fig 3 in S2). These findings were also supported by rarefaction curves, where most of the body skin samples had a higher slope than the curves of the ear canal samples indicating the presence of more complex bacterial communities on the body skin sites (Fig 1c).

**Fig 1.** Box-plots of alpha diversity indices. Box-plots of alpha diversity indices of Shannon (a) and Chao 1(b) and Rarefaction curves ((c); specimens versus taxa) of the non-allergic body skin (axilla, interdigital, groin; AINTL) vs the non-allergic ear canal. The sample sites are arranged as the curves in descending order and with an equivalent color. Asterisks are representing statistical significance: \*\*\* p < 0.001.

Skin microbial composition of non-allergic GSDs

Bacterial community patterns present at the different body sites for the individual non-allergic dogs were investigated by NMDS analysis based on a similarity matrix generated with the Bray Curtis similarity index. One way ANOSIM analysis (p= 0.17) and One way PERMANOVA analysis (p= 0.1808) did not reveal significant differences between the four different body sites (axilla, interdigital, groin and ear canal) of the non-allergic GSDs. A variability across all dogs and all different body sites was documented and the factor home had the strongest impact on the bacteria community composition (largest relative length of biplot vector; Fig. 2). No significant difference was found for the three body sites examined as body skin (axilla, interdigital, groin) versus ear canal (One way ANOSIM p= 0.8903; One way PERMANOVA p= 0.3098). However, significant differences in community composition were identified when grouping by sex (male versus female; One way ANOSIM and One way PERMANOVA p<0.001; R=0.1337) was conducted. Significant differences in the bacterial community composition were also observed when grouped by homes (both One way ANOSIM and One way PERMANOVA p<0.003; R=0.1337; Fig 2 in S2; homes Table 1 in S1).

**Fig 2.** Comparative analysis of the bacterial community composition of the skin microbiota of all non-allergic dogs.Comparative analysis of the relative abundance patterns of the skin microbiota composition of all body sites (different objects), performed by NMDS analysis based on a Bray-Curtis similarity matrix. The sex, body site, and home of living were included as environmental parameters (biplots).

Composition (most abundant taxa) of the skin of non-allergic GSDs

Forty-four phyla in total were identified from the four sample sites (axilla, interdigital, groin, ear canal) taken from the non-allergic dogs, of which 30 phyla had a relative abundance lower than 1%. The ten most abundant phyla of non-allergic dogs were Actinobacteria (mean relative abundance 29.1 ±14.7%) followed by Proteobacteria(27.4 ±10.1%), Firmicutes (20.3 ±18.7%) and Bacteroidetes (10.8 ±5.4%), Cyanobacteria, Acidobacteria(both 2.0 ±1.6%), Chloroflexi (1.8 ±1.6%), Planctomycetes (1.7 ±1.4%), Deinococcus-Thermus (1.5 ±1.2%) and Verrucomicrobia (1.1 ±1.0%) (Fig 3a). In samples from the interdigital region and the ear canal Proteobacteria were most abundant followed by Actinobacteria, Firmicutes, Bacteroidetes and Cyanobacteria*.* Groin samples were dominated by Actinobacteria followed Proteobacteria, Firmicutes, Bacteroidetes and Cyanobacteria. Ιn the samples from the axilla Firmicutes was most abundant followed by Actinobacteria, Proteobacteria, Bacteroidetes and Cyanobacteria (Fig 3a; Table 5 in S1).

Analysis of the mean relative abundance at the genus level of the most abundant phylogenetic groups colonizing the different body sites (Table 6 in S1) revealed some important differences (Table 7 and Table 8a in S1). The skin of the axilla was mostly colonized by *Macroccocus* (mean relative abundance 13.8 ± 29.8%)followedby *Brevibacterium*, *Staphylococcus*, *Clostridium sensu stricto 7*, *Nocardioides, Sphingomonas* and others. The interdigital area was dominated by *Clostridium sensu stricto 7* (mean relative abundance 3.5 ± 2.7%) followed by *Nocardioides*, *Pelomonas*, *Sphingomonas*, *Vibrionimonas*, *Psychrobacter*, *Bravibacterium* and *Staphylococcus*. The groin was mostly colonized by *Staphylococcus* (mean relative abundance 10.8 ± 25.5%), followed by *Corynebacterium1*, *Conchiformibius*, *Nocardioides*, *Brevibacterium*, *Macrococcus* and *Porphyromonas*. *Brevibacterium* had the highest mean relative abundance (3.7 ± 7.6%) in the ear canal followed by *Clostridium sensu stricto 7*, *Sphingomonas*, *Psychrobacter*, *Pelomonas*, *Flavobacterium*, *Deinococcus* and *Pseudomonas*. Subsequently, each of the four taxa with the highest mean relative abundance per body site (axilla: *Macroccocus;* interdigital: *Clostridium sensu stricto 7;* groin: *Staphylococcus;* ear canal: *Brevibacterium*) was evaluated individually for significant different abundance between the four body sites. None of these taxa had a significantly different relative abundance between the four body sites (normality failed; Kruskal-Wallis-test for axilla vs interdigital vs groin vs ear canal; *Macroccocus*: p=0.975; *Clostridium sensu stricto 7*: p= 0.085; *Staphylococcus*: p= 0.288; *Brevibacterium*: p= 0.406).

**Fig 3.** Phylogenetic composition of the skin and ear canal of non-allergic versus allergic dogs. (a) Relative abundance of the 10 most common bacterial phyla identified in axilla, interdigital, groin and ear canal in non-allergic and allergic dogs. (b) Variation of the relative abundances of the 10 most abundant phyla among non-allergic and allergic dogs. Asterisks are representing statistical significance: \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001.

Alpha diversity of bacterial communities present at skin samples of allergic GSDs

Richness (Chao1) and diversity (Shannon) of phylogenetic groups at the four sites (axilla, interdigital, groin, ear canal) of allergic GSDs were not significantly different between the body sites (Kruskal-Wallis for Shannon index, p= 0.334; for Chao 1, p= 0.78).

Skin microbial composition of allergic GSDs

The bacterial community patterns of the different body sites for the individual allergic dogs were investigated by NMDS analysis based on a similarity matrix generated with the Bray Curtis similarity index. One way ANOSIM analysis (p= 0.6) and One way PERMANOVA analysis (p= 0.32) did not reveal significant differences between the four different body sites (axilla, interdigital, groin and ear canal) of the allergic GSDs.

In order to evaluate if oclacitinib had an effect in the composition of the skin microbiota of the allergic dogs, we compared the bacterial communities’ composition, as described above for the subgroups allergic with oclacitinib (n=6) and without oclacitinib (n=6). No significant difference was obtained, proposing that oclacitinib did not affected the cutaneous and ear canal microbiota composition (One way ANOSIM p-values; axilla: p= 0.2777; interdigital: p= 0.2949; groin: p= 0.1494; ear canal: p= 0.9022) among the allergic dogs, given the small number of the subgroup population. On this basis we assume that oclacitinib should not affect the comparison between allergic and non-allergic dogs.

Composition (most abundant taxa) of the skin of allergic GSDs

Proteobacteria(mean relative abundance 35.5 ±10.9%) and Actinobacteria(22.4 ±10.2%) were the most abundant phyla of the allergic GSDs and dominated in each of the four sample sites (Figs 3a and 3b; Table 11 in S1). They were followed by Firmicutes (17.0 ±14.6%), Bacteroidetes(14.1 ±5.2%), Cyanobacteria (2.5 ± 3.0%), Planctomycetes (1.8 ±1.2%), Chloroflexi (1.7 ±1.5%), Acidobacteria (1.7 ±1.6%), Verrucomicrobia (0.7 ± 0.9%) and Deinococcus-Thermus (0.6 ±0.7%) (Figs 3a and 3b; Table 11 in S1).

*Sphingomonas* and *Staphylococcus* dominated the skin microbiota of allergic GSDs, followed by *Clostridium sensu stricto 7, Nocardioides* and others(Tables 8a and 8b in S1; Fig 8). Analysis of the mean relative abundance at the genus level of the most abundant phylogenetic groups colonizing the different body sites (Table 6 in S1) revealed some important differences for the allergic GSDs too (Table 7 and Table 8a in S1). *Clostridium sensu stricto 7* (mean relative abundance 6.2 ±7.8%) was the most abundant on the skin of the axilla and was followed by *Staphylococcus*, *Vibrionimonas*, *Sphingomonas*, *Pelomonas*, and *Nocardioides*. The interdigital skin was dominated by *Nocardioides* (mean relative abundance 5.3 ±4.4%), followed by *Sphingomonas*, *Pelomonas*, *Clostridium sensu stricto 7*, *Vibrionimonas*, *Bradyrhizobium*, *Staphylococcus*, and *Hymenobacter*. The skin of the groin was dominated by *Sphingomonas* (mean relative abundance 4.9 ±2.4%), followed by *Clostridium sensu stricto 7*, *Nocardioides*, *Porphyromonas*, *Conchiformibius*, *Pelomonas*, and *Vibrionimonas*. Finally, the ear canal of the allergic GSDs was dominated by *Staphylococcus* (mean relative abundance 9.4 ±25.9%), followed by *Sphingomonas*, *Clostridium sensu stricto 7*, *Nocardioides*, *Vibrionimonas*, *Pelomonas*, *Propionibacterium*, and *Paracoccus*.

Differences in the skin Microbiome between non-allergic and allergic German shepherd dogs

Diversity and richness of skin microbiota of allergic versus non-allergic GSDs

Comparing the diversity between allergic and non-allergic GSDs a significantly lower bacterial community richness was determined at the skin of the axilla of allergic GSDs (A; Chao 1 index; p= 0.032; Fig 5a). This finding was supported by respective rarefaction curves showing that the samples from the axilla of non-allergic dogs had a higher slope than the curves from the samples of allergic dogs indicating the presence of more complex bacterial communities in non-allergic dogs (Fig 6). The evenness of the skin microbiota in the groin of allergic dogs was significantly lower because of the high abundance of individual phylogenetic groups (Evenness index values, Table 9 in S1; Fig 4 in S2). Shannon and Dominance indices were not significantly different (Table 9 in S1).

**Fig 4.** Box-plots of alpha diversity index Shannon (total diversity) of the skin microbiota per body site (axilla: (a); interdigital (b); groin (c); ear canal (d)) of non-allergic and allergic German shepherd dogs, based on Illumina 16S rRNA gene amplicon sequencing of microbial communities.

**Fig 5.** Box-plots of alpha diversity index (number of taxa considering the number of singletons) per body site (axilla: (a); interdigital (b); groin (c); ear canal (d)) of non-allergic and allergic German shepherd dogs using Chao 1 index,based on Illumina 16S rRNA gene amplicon sequencing of microbial communities. Asterisks are representing statistical significance: \* p < 0.05.

**Fig 6.** Rarefaction curves (specimens versus taxa) based on Illumina 16S rRNA gene amplicon sequencing of microbial communities from the axilla (A) of allergic (a-) and non-allergic GSD. The sample sites are arranged as the curves in descending order and with an equivalent color.

Differences in the composition of bacterial communities of allergic versus non-allergic GSDs

Shifts in the bacterial community composition at the skin of allergic dogs compared to non-allergic dogs were visualized in NMDS plots (Fig 7). Significant differences were obtained for the axilla (A; One way ANOSIM p=0.048, R=0.1001; One way PERMANOVA p=0.0256), groin (L; One way ANOSIM p=0.036, R=0.00386; One way PERMANOVA p= 0.0264) and ear canal (O; One way ANOSIM p=0.0012, R=0.1842; One way PERMANOVA p= 0.0025), but not for the interdigital area (Int; One way ANOSIM p=0.1548; One way PERMANOVA p= 0.1171). The inclusion of the health status (non-allergic vs allergic) confirmed that allergy correlated with the significant differences of the clustering of the community patterns between the samples (Fig 7). Sample a18O (ear canal of the allergic dog a18) was excluded from the NMDS plot because the community was too different (Fig 5 in S2). The strong difference of the community profile was due to the high relative abundance of the genus *Staphylococcus* (91.3%) compared to 0.02-6.4% in the other samples (Table 6 in S1).

In order to determine which taxa (phylogenetic groups/genera) had the main contribution to the community differences among allergic versus non-allergic GSD for each body site, SIMPER analysis was performed. Phylogenetic groups with the highest contributions (>2%) are listed in S1 Table 10. The genus *Macrococcus* (9.0% contribution) was the major contributor for the differences in the samples from the axilla (A), followed by *Staphylococcus* (4.7% contribution). For the samples from the groin (L) the genus *Staphylococcus* contributed the most (7.0% contribution). For the ear canal (O), *Brevibacterium* contributed the most to the differences (2.5% contribution) with S*taphylococcus* of the sample a18O being excluded as dominated all the other, as mentioned above, affecting the comparison.

**Fig 7.** Comparative analysis of the skin microbiota composition of relative abundance patterns at each body site of non-allergic (black color) and allergic (red color) dogs, performed by NMDS analysis based on a Bray-Curtis similarity matrix. The health status was included as environmental parameter; (A- Axilla, Int- interdigital area, L- groin, O- ear canal)

Composition (most abundant taxa) of the skin of allergic versus non-allergic GSDs

The order of the 10 most abundant phyla of allergic GSDs was different compared with non-allergic GSDs (Table 5 and Table 11 in S1). Between the two groups, Proteobacteria (p< 0.001) and Bacteroidetes(p= 0.003) had a significantly higher mean relative abundance at the skin of allergic dogs, whereas Actinobacteria (p= 0.012), Deinococcus-Thermus (p< 0.001) and Verrucomicrobia *(*p= 0.016) occurred in a significantly lower relative abundance in allergic dogs. Firmicutesshowed no significant differences between allergic and non-allergic GSDs (Fig 3b; Table 11 in S1).

Evaluating the mean relative abundances of the phylogenetic groups for significant differences between allergic and non-allergic GSDs showed that *Sphingomonas* was significantly higher abundant in the allergic GSDs (p<0.001;mean rel. abundance of non-allergic 2.5 ±1.4% versus 4.9 ±2.3% of allergic dogs), as well as *Nocardioides* (p=0.034; mean rel. abundance of 2.5 ±1.8% for non-allergic dogs versus 4.3 ±3.4% for allergic dogs). No significant difference was observed for *Staphylococcus* (p=0.8, Table 7) and *Clostridium sensu stricto 7* (p=0.062) between allergic and non-allergic GSDs. Interestingly, allergic dogs had significantly lower mean relative abundance of *Macrococcus* (p<0.001; mean rel. abundance of 4.8 ±15.5% for non- allergic dogs versus 0.1 ±0.4% for allergic dogs; Table 12b in S1) and *Brevibacterium* (p= 0.016; mean rel. abundance of 2.7 ±6.3% for non- allergic dogs versus 0.1 ±0.3% for allergic dogs) than non-allergic GSDs.

A more detailed comparison between the allergic and non-allergic GSDs of the most abundant taxa per body site revealed important findings. *Clostridium sensu stricto 7* occurred in a significantly higher relative abundance on the axilla of allergic dogs (p= 0.026;mean relative abundance of 2.7 ±5.0% for non-allergic dogs versus 6.2 ±7.8% for allergic dogs). *Nocardioides* of the interdigital skin showed no significant difference (p= 0.138). *Sphingomonas* was significantly more abundant in the groin of the allergic dogs (p= 0.002; mean relative abundance of 2.0 ±1.5% for non-allergic dogs versus 4.9 ±2.4% for allergic dogs). In addition, *Sphingomonas* was significantly more abundant in the allergic versus non-allergic GSDs for multiple sites (axilla: p= 0.013; interdigital: p= 0.014; ear canal: p= 0.017; Fig 8; Table 8 in S1). *Staphylococcus* did not show any significant difference for any site between the allergic and non-allergic GSDs (Table 12a in S1). On the contrary, a significantly lower relative abundance of *Macrococcus* was obtained from samples of the interdigital skin, the groin and the ear canal of the allergic dogs but there was no significant difference for the axilla (Table 12b in S1). In addition, *Brevibacterium* was also evaluated for significant difference for the ear canal samples because it was the main contributor to the difference of the bacterial community composition between the two groups in SIMPER analysis, with the allergic group having a significantly lower mean relative abundance (p= 0.041; mean relative abundance of 3.6 ±7.7% for the non-allergic dogs versus 0.1 ±0.4% for the allergic dogs).

**Fig 8.** Relative abundance of the 10 most abundant taxa of non-allergic and allergic dogs, from across all body sites. Asterisks are representing statistical significance: \* p< 0.05; \*\* p< 0.01; \*\*\* p< 0.001.

Discussion

In this NGS study, we described the bacterial community of the skin and ear canal of non-allergic GSDs and compared it to the skin and ear canal microbiota of allergic GSDs. Several NGS studies have also examined the canine skin or ear canal in health and disease. These studies used different techniques, from sample collection and storage, to DNA extraction and analysis and as shown before, methodology influences results [43–45]. Therefore, a direct side by side comparison of studies is difficult [43]. Nevertheless, any of those results are useful to further characterize the canine cutaneous microbiota in health and disease. Our 16S rRNA gene amplicon approach (nested PCR; V3-V6 & V4-V5) showed high individual and body site variability between the different study subjects, without significant differences between the body sites, in contrast to the first NGS-based study in veterinary medicine [13]. In our study the body skin (axilla, interdigital, groin) showed significantly higher species richness than the ear canal in non-allergic dogs. Possible explanations may include the special ear canal construction (chamber-like) and histology, with a comparatively thin epidermis and dermis containing single hair follicles, sebaceous glands and ceruminal glands, providing lipid-rich cerumen determining it´s microenvironment with a relatively high humidity [46,47]. Bactericidal activity of cerumen could explain the lower species richness compared to the other body sites.

We identified Actinobacteria as the most dominant phylum on the non-allergic dogs, similar to another study [48]. This finding is also in line with the bacterial composition of human skin [3]. In contrast, the first NGS veterinary study describing healthy canine cutaneous bacterial composition showed predominately Proteobacteria [13]. As mentioned above, different methodology could explain such differences as studies have shown [43,45,46,49]. Thus, this phenomenon is not unique to dogs. Furthermore, studies have shown that environmental factors can influence the skin microbiome in humans like contact with soil or plant material [48,50–52]. Therefore, it can be assumed that different habits or environment of the dogs could explain differences in results. In addition, temporal changes of microbiome can also influence the results and lead to such disagreements between studies. To date temporal stability of cutaneous microbiome in animals remains largely unknown. In rats healthy skin showed temporal instability (11 days) [53], whereas the healthy canine ear canal remained stable for 28 days. [1]. Both, living in the same household and sex affected the bacterial community patterns among the samples of the healthy dogs, in agreement with other studies [48,54]. Interestingly in our study, Proteobacteria predominated in the ear canal and the interdigital area, as in another study for the interdigital area and the pinna [13]. It is not clear why these two areas showed this difference in comparison with the groin and the axilla of the GSDs. One possible explanation for the interdigital area could be direct and repeated contact with soil, where it is shown that Proteobacteria predominate its bacterial composition [55]. Currently there are only two other NGS studies describing the canine ear canal. In the first study, Proteobacteria were the most abundant phyla followed by Actinobacteria, Firmicutes, Bacteroidetes and Fusobacteria with *Escherichia* as the most abundant genera [1]. The second study showed also similar most abundant phyla but in a different order, with Firmicutes being most abundant followed by Proteobacteria, Bacteroidetes and Actinobacteria with *Romboutsia* as the most abundant genus [56]. Our findings were consistent with the first study [1], as we had the same order of the most common phyla, except that we documented Cyanobacteria instead of Fusobacteria. At the genus level we identified *Brevibacterium* as the most abundant taxon. The most abundant genus found on non-allergic skin was *Macrococcus* with the highest abundance in the axilla. The second most common genus was *Staphylococcus* with the highest abundance in the groin followed by the axilla, the interdigital area and the ear canal. *Clostridium sensu stricto 7* was the most common genus of the interdigital area. In contrast, one previous study found that healthy canine skin (axilla, pinna and groin) and mouth was predominantly colonized by *Porphyromonas*, *Staphylococcus*, *Streptococcus* and *Propionibacterium*, with *Porphyromonas* significantly colonizing the axilla [14]. In another study, analysis of healthy canine skin (dorsal nose, nasal mucosa, lip commissure, conjuctiva, periocular skin, ear canal, concave pinna, dorsal lumbar area, axilla, groin, interdigital skin, and perianal area), revealed *Ralstonia* as the most abundant genus in most of the samples [13]. A recent exploratory study with six healthy dogs showed that on the skin (inguinal, axilla, periocular and trunk), *Pseudomonas* was most abundant followed by *Kocuria*, *Porphyromonas* and *Corynebacterium* [57].While another one identified *Propionibacterium* *acnes*, *Corynebacterium* and *Porphyromonas* as most abundant in the skin (dorsal neck, axilla, and abdomen) of 40 healthy dogs [48]. These findings are in contrast to our study. *Macrococcus* is a Gram-positive coccoid bacterium, previously placed into the *Staphylococcus* genus but since 1998 assigned to its own genus [58]. It is composed of eight species that are closely related with species of the genus *Staphylococcus* [58]. Current information on the distribution of *Macrococcus* is limited. This genus is described primarily as part of the microbiota of mammals and in milk and meat according to a current review paper [58]. Even though it is considered as a non-pathogenic bacterium, there are a few reports of infections associated with *Macrococcus caseolyticus* and *M. canis* [59–62]. A recent study found that of 162 dogs, 13 carried *M. canis* and six *M. caseolyticus* predominately in cutaneous (axilla and groin) non-infectious sites. Six *M. canis* and one *M. caseolyticus* strains were isolated from animals with rhinitis, otitis externa, dermatitis and mastitis [59]. As both healthy and infected skin was colonized, it was concluded that *Macrococcus* is an important opportunistic bacterium of the canine skin [59]. Because *Macrococcus* contributed mainly to the difference in bacterial community composition of the axilla between non-allergic and allergic GSDs, and was the genus with the highest relative abundance found on the skin of non-allergic dogs a potential protective role can be speculated too. Thus further studies regarding the distribution and role of bacteria of this genus in healthy and diseased dogs are needed.

An important finding of our study was that the site-specific bacterial composition significantly differs with allergic skin disease. The SIMPER analysis revealed the taxa with the major contribution to these changes, even though the mean relative abundance of some of those taxa (e.g. *Staphylococcus*) did not differ significantly between non-allergic and allergic GSDs. A possible explanation for this is that SIMPER analysis identified genera that had the highest contribution to differences between the groups without being necessarily significantly different. Furthermore, the axilla of allergic dogs showed a significantly decreased diversity (species richness) indicating dysbiosis (due to higher relative abundance of *Clostridium sensu stricto* 7 and *Sphingomonas*), even though the atopic dogs showed no clinical flares and had no pyoderma at the time of the sampling. This is consistent with another study of atopic dogs with pyoderma, which demonstrated different bacterial communities and reduced diversity in the allergic dogs [14]. The first 16S rRNA gene amplicon based NGS study using allergic dogs without flares, like our study population, did not show significant differences of the skin microbiota community composition in comparison with healthy dogs in contrast to our findings, but did show that allergic skin had lower diversity (richness), similar to our findings [13]. The ear canal of allergic dogs without signs of otitis also had a bacterial composition significantly different from healthy dogs’ ear canal, and without a significant difference in diversity between the groups, similar to our results [1]. A trend of dysbiosis in association with allergy was shown with significantly increased abundance of *Staphylococcus* and *Ralstonia* in the atopic ear canals [1]. In general, we were able to show a trend of dysbiosis for all the sites evaluated in total, with significantly reduction of *Macrococcus* and *Brevibacterium* and increase of the phylum Proteobacteria and the genera of *Sphingomonas* and *Nocardioides*.

Six out of 12 allergic dogs received oclacitinib, which is a Janus kinase inhibitor with anti-pruritic and anti-inflammatory properties [63]. Until now there is no study investigating the impact of oclacitinib on the skin microbiota. One study documented that treatment with cyclosporine or corticosteroids did not affect the cutaneous microbiota in dogs, evaluating these dogs before, during and after treatment [64]. This is in line with our findings, bacterial composition of allergic dogs with and without oclacitinib did not show any significant differences. Thus, we suggest that oclacitinib may not influence the overall comparison between the non-allergic and allergic dogs, but future studies with larger population should confirm our finding.

One limitation of our study was the small sample size. However our sample size is larger than in previous studies [1,13,57,64]. Furthermore, our study adds to a growing corpus of research that helps us better understand the microbiota inhabiting the skin and ear canal of dogs and can be used to design larger confirmatory studies. The study was a cross-sectional analysis and therefore it remains unclear if findings are a cause or a result of allergy. Regarding the description of the cutaneous microbiota only a longitudinal study could clarify if the composition of the microbiota is stable or only transient. Further studies including also non-allergic dogs from other breeds are required, in order to evaluate if the skin microbiota of non-allergic GSDs is breed specific or not. Because previous studies of humans’ skin microbiota detected a low amount of microbial DNA when standard PCR was used, a nested PCR was chosen in our study [24,25]. A bias in alpha diversity and community structure has been documented due to a nested PCR in stool samples but not in vaginal swabs and might be considered as a possible limitation factor of our study [65]. Currently, no standard protocol exists for the methodology, and as consequence different primers are used in different studies [43]. Because there is very limited systematic comparison of the primers, and no "perfect" primer exists [66], most commonly, primers are selected based recommendations and the experimental experience of the laboratory [49]. Our second primer system targeted the V4-V5 hypervariable region, which is currently used for 16S rRNA gene sequencing-based microbial profiling [49,67,68]. Our choice was based on studies that suggest an accurate estimation of multiple taxa and an efficient phylogenetic resolution with this hypervariable region [49,67]. These advantages, were again demonstrated recently [69]. On the other hand, as it was shown in the same study, targeting this region (or targeting other regions) might have an effect on the result, because different primers do not always detect as expected specific bacterial communities, resulting in a bias when quantification of those communities is assessed [69]. But the primer system amplifiing the V4-V5 was one of the primer systems with was less biases, and probably these effects may be subtle [69]. It was also shown that the analysis of bacterial community composition (betadiversity studies) were robust for the used of different primers [66,69]. Two allergic dogs with mild skin lesions (CADESI-4 score: 2 and 4) had a PVAS score of 8/10 and 7/10, possibly due to an initiating flare state. In order to minimize the possibility to include a dog with a subclinical cAD into the non-allergic group, the age of the healthy dogs had to be more than four years old. Consequently, the age of dogs of two groups was significantly different. This might be a limitation, as it has been shown in humans that the skin microbiota evolve with age (first years after birth or with sexual maturation) [70–72]. In addition, all dogs were living in the same area of Germany and the environment affects skin microbiota [73], possibly leading to differences between results of studies from other countries. In our study, we did not evaluate the association of rural or urban environment with the dogs’ cutaneous microbiota. A last limitation of our study is that the 16S rRNA gene sequencing cannot differentiate dead from alive bacteria [2]. Larger studies including other breeds, several countries, as well as dogs with and without allergy flares could elucidate the canine skin microbiota in health and disease.

Conclusion

This study describes and compares the cutaneous and ear canal microbiota of non-allergic and allergic GSDs, showing that allergic dogs have a significantly different bacterial community composition and significantly lower species richness (alpha diversity). The skin and ear canal microbiota of non-allergic GSDs is highly variable, and the ear canal has a significantly lower species richness than the body skin.

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**Conflict of interest:**

One of the authors (UM) is employed by a commercial company (AniCura Kleintierspezialisten Augsburg GmbH). This company provided the salary for the clinical duty of the author (UM). Her clinical duty is not dependent on the study. Furthermore, the company did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific role of these author is articulated in the ‘author contributions’ section. This commercial affiliation does not alter our adherence to PLOS ONE policies on sharing data and materials

Supporting information

S0 Table 1-3

S1 Tables 1 - 12

S2 Figs 1 - 5

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