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## 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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Samples were collected 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.

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Dear Plos One Editor,

herewith we re-submit our manuscript with the title "Description and comparison of the skin and ear canal microbiota of non-allergic and allergic German shepherd dogs using next generation sequencing". We performed 16S rRNA gene amplicon sequencing of DNA samples extracted from swap samples taken from the skin of German Shepard dogs.
We provided now a clear statement that an ethical approval was not required. Samples were collected 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. The respective statement is now given in the material and method part on page 4 paragraph "Study subject".

In addition, we added one table to S1 (Table 6), which was missing before. The table shows the relative abundances of all detected bacterial taxa in the individual samples.
The master student Ruchi Bagwe was now moved from the Acknowledgment to the authors after she was reading the paper and conformed the co-authorship.

Best regards, Stefanie Glaeser

# 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 canal of healthy non-allergic German shepherd dogs (GSDs) and to compare it with the skin microbiota of allergic GSDs without acute flare or concurrent skin infection. Bacterial 16S rRNA gene amplicon sequence data revealed no differences of bacterial community pattern between the different body sites in non-allergic dogs. The ear canal microbiota of non-allergic German shepherd dogs had a reduced diversity compared to the body 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 bacteria microbiota of allergic dogs were characterized by a site specific (axilla) lower species richness compared to non-allergic dogs. *Actinobacteria* was the most abundant phylum identified from the non-allergic dogs and *Proteobacteria* from allergic dogs. *Macrococcus* spp. were more abundant on non-allergic skin while *Sphingomonas* spp. was more abundant on the allergic skin.

## Introduction

Several next generation sequencing studies (NGS) 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

73 composition [1]. All previously mentioned studies involved dogs from various breeds.

74 It is well documented that cAD has breed predispositions and that the phenotype of

75 the disease differs between breeds [15]. It is unclear if the different phenotypes of cAD

76 affect the bacterial community composition between the breeds. To date no study has

77 evaluated the microbiota of the skin and ear canal of only a single breed in both healthy

78 and allergic skin disease states, thus minimizing potential bias due to allergy

79 phenotype effect on the microbiota. The German shepherd dog (GSD) was chosen in

80 our investigation because it is a high-risk breed for cAD [15], possibly due to an altered

81 expression of the plakophilin 2 gene and other genes of the chromosome 27 [16].

82 The goal of the study was to describe and compare bacterial microbiota of four

83 body sites (axilla, front dorsal interdigital skin, groin and ear canal) of healthy (non-

84 allergic) and allergic GSDs using a 16S rRNA gene amplicon based Illumina

85 sequencing approach. We hypothesized that atopic dermatitis and/or CAFR influence

86 the microbiota of the skin and ear canal of GSDs resulting in reduced bacterial diversity

87 and significantly different bacterial community composition.

88

## Material and methods

## Study subjects

91 The clinical study was performed at the small animal clinic of the Justus-Liebig

92 University, Giessen, Germany. Samples were collected during routine diagnostic

93 appointments specifically for this study. All owners were informed of the procedures

94 and signed a consent form for sample collection. The Animal Welfare Committee of

95 the Justus-Liebig University of Giessen was informed about the study protocol and

96 especially the sampling method was discussed. As there is neither pain, harm nor

97 damage caused by gently rolling a cotton swab on skin, they assured us that ethical

4

98    approval by the responsible authority is not required. Two groups of GSDs were

99    studied. The control group included 12 non-allergic GSDs without any history of allergic

100   conditions or any clinical skin/ear canal lesions compatible with allergy at the time of

101   examination and sampling. In order to minimize the risk of including a dog with

102   subclinical allergy, only dogs older than four years old were involved, as allergic

103   conditions most often develop in young dogs from 6 months to 3 years [17]. In order to

104   investigate a possible influence of the household conditions to the microbiota, two non-

105   allergic GSDs per household were required. Neither systemic antibiotics nor any

106   immunomodulatory or anti-inflammatory drugs were allowed six months prior to

107   sampling. Bathing with shampoo and the use of ear cleaners was not allowed seven

108   days prior to sampling. Twelve adult allergic GSDs were diagnosed with cAD, either

109   due to cutaneous food reactions or environmental allergens. Standard diagnostic and

110   therapeutic methods were used, including fulfillment of at least five of Favrot´s criteria

111   and excluding other pruritic dermatosis (e.g. flea saliva hypersensitivity, sarcoptic

112   mange) [12,18]. A combination of cAD and/or CAFRs with flea saliva hypersensitivity

113   was allowed. Dogs with any secondary bacterial or fungal skin or ear infection were

114   excluded. Systemic and topical antibacterial or antifungal agents were not allowed 30

115   and 14 days prior to sampling, respectively. Systemic administration of any

116   immunomodulatory or anti-inflammatory drugs, with the exception of oclacitinib, were

117   equally not allowed two months prior to sampling. Topical immunomodulatory or anti-

118   inflammatory drugs had to be withdrawn 14 days prior to the study. Any shampooing

119   and ear cleaners were not allowed seven days prior to sampling. All owners filled out

120   a questionnaire regarding their dog's housing, partner animals, current diseases and

121   treatment, food supplements, frequency of bathing and type of shampoo. Owners of

122   allergic dogs were asked about gastrointestinal signs and to score their dog's pruritic

123   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 for 10 times. All samples were obtained in duplicates. Subsequently, a sample was taken for cytological purposes using a sterile cotton swab.

149    The samples were transported immediately at 8°C to the Institute of Applied

150    Microbiology, JLU Giessen, for storage and further processing. Samples were stored

151    at -20°C until all samples were collected.

152

## DNA extraction and 16S rRNA gene amplicon sequencing

154    Total DNA was extracted from DNA free swaps using the NucleoSpin® 96 Soil

155    kit (96-well extraction system, Macherey Nagel AG, Oesingen, Switzerland) which can

156    efficiently extract DNA from Gram-negative and Gram-positive bacteria including

157    spores. According to manufacturer's instruction using vacuum processing (NucleoVac

158    96 vacuum manifold, Macherey Nagel AG, Oesingen, Switzerland) with slight

159    modifications, extraction was started using lysis buffer SL1 and afterward steps 1 to 5

160    were repeated with lysis buffer SL2, thus samples were lysed twice. Total DNA was

161    eluted with approximately 80 µL PCR water (1x 30 µL, 1x 50 µL) instead of SE buffer.

162    DNA was quantified spectrophotometrically using a NanoDrop spectrophotometer

163    (Thermo Scientific) and subsequently checked for the presence and amplifiable 16S

164    rRNA gene sequences for selected samples.

165    The 16S rRNA gene sequences of *Bacteria* were amplified for Illumina amplicon

166    sequencing (LGC Genomics, Berlin, Germany) using a nested PCR approach with a

167    first PCR with the primer system - 341F/1061R (V3-V6) (20 cycles) followed by a

168    second PCR with primer system 515F-Y and 926R-jed (V4-V5) (20 cycles), because

169    according to previous studies, only a low amount of microbial DNA was detected on

170    human skin analyzed by PCR [24,25]. For each sample, the forward and reverse

171    primers had the same 10-nt barcode sequence. The first round of PCR was carried out

172    for 20 cycles, using the following parameters: 2 minutes 96°C pre-denaturation; 96°C

173    for 15 seconds (s), 50°C for 30 s, 70°C for 90 s and primers without inline barcodes

174    were used (341F/1061R). For the second round, 1 µl PCR product from the first round

175 was used and the PCR conditions were the same as before. In this case, barcoded

176 primers were added (515F-Y/926R-jed). DNA concentration of amplicons of interest

177 was determined by gel electrophoresis. About 20 ng amplicon DNA of each sample

178 were pooled for up to 48 samples carrying different barcodes. The amplicon pools were

179 purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and

180 other small mispriming products, followed by an additional purification on MiniElute

181 columns (Qiagen). About 100 ng of each purified amplicon pool DNA was used to

182 construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96

183 (NuGEN). Illumina libraries were pooled, and size selected by preparative gel

184 electrophoresis. Sequencing was done on Illumina MiSeq using V3 Chemistry

185 (Illumina). Raw sequence reads are available in the Sequence Read Archive (SRA)

186 with BioSample Accession numbers SAMN14565128 to SAMN14565223 in the

187 BioProject PRJNA624030.

188 Using the NGS analysis pipeline (https://www.arb-silva.de/ngs) of the SILVA

189 rRNA gene database (SILVAngs 1.3) combined sequence reads were processed [26].

190 All reads were aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB

191 SVN (revision21008)) [27], against the SILVA SSU rRNA SEED and quality controlled

192 [26]. Reads shorter than 50 aligned nucleotides and reads with more than 2% of

193 ambiguities, or 2% of homopolymers, were excluded from further processing. Reads

194 with a low alignment quality (50 alignment identity, 40 alignment score reported by

195 SINA) as well as putative contaminations and artifacts, were identified and excluded

196 from downstream analysis. The next process step was dereplication and clustering

197 with cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) using *accurate*

198 *mode*, ignoring overhangs and applying identity criteria of 1.00 and 0.98 respectively

199 [28]. For classification a local nucleotide BLAST search was performed against the

200 non-redundant version of the SILVA SSU Ref dataset (release 128; http://www.arb-

201 silva.de) using blastn (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with

202 standard settings [29]. Unique reads were clustered in operational taxonomic unit

203 (OTU) on a per sample basis under the criterium of 98% sequence identity to each

204 other (pairwise distance and single linkage clustering). The longest read in each cluster

205 was classified as the reference for each OTU and was mapped onto all reads that were

206 assigned to the respective OTU. This process resulted in quantitative information

207 (number of individual reads per taxonomic path), despite the PCR limitations, possible

208 sequencing technique biases and multiple rRNA operons. Reads without any BLAST

209 hits or reads with weak BLAST hits, where the function "(% sequence identity + %

210 alignment coverage)/2" did not exceed the value of 93, remained unclassified and were

211 assigned in the virtual taxonomical group "No Relative" in the SILVAngs fingerprint and

212 Krona charts [30], as previously reported [31,32].

213

214 **Statistical analysis**

215 Statistical analysis was perfromed in PAST3 (https://folk.uio.no/ohammer/past)

216 [33]. Absolute and relative abundance data of sequences of present phylogenetic

217 groups (taxa) were used. The analysis was performed at the level of phylogenetic

218 groups, which was given by a resolution at the genus level. Alpha diversity was

219 measured by calculating different diversity indices, such as Shannon (overall α-

220 diversity), Chao 1 (richness; number of taxa corrected by the presence of singleton),

221 Dominance (=1-Simpson index; to see if one taxon is dominating or if all taxa are

222 equally distributed, Evenness (Buzas and Gibson´s evenness: eH/S) [34] and

223 rarefaction curve analyses considering the number of taxonomic paths and the number

224 of reads per taxonomic path to evaluate richness in different sample sizes [35].

225 Significant differences between the alpha diversity indices were further evaluated. For

226 two groups/communities, i) body skin (axilla, interdigital, groin) vs ear canal or ii) a

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 [36]. 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 [37]. The phylogenetic composition of the bacterial communities were studied based on relative abundance data analyzed by non-metric multidimensional scaling (NMDS; [38]) using Bray-Curtis similarity matrix [39]. 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 [40] and One way PERMANOVA [41] were performed to significant differences (evaluating the sequential Bonferroni p-values) among samples. SIMPER (Similarity Percentage) analysis [40] 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 sprayed 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

252    of max 131 kilometers away from the town of Giessen (Germany). The age of the non-

253    allergic dogs (mean: 7.5 ±1.9 years) was significantly higher (t-Test p< 0.01) than the

254    age of allergic GSDs (mean: 4.8 ±2.2 years). All allergic dogs had mild lesions with a

255    median CADESI-04 score of six (range 0-11) and a median PVAS score of four (range

256    0-8) (Table 2 in S1). Two dogs had a PVAS score of 7/10 and 8/10, respectively with

257    only mild skin lesions. Six allergic GSDs received labeled doses of oclacitinib (Apoquel,

258    Zoetis Deutschland GmbH, Berlin, Germany).

259

## Analysis of the phylogenetic composition of the GSDs microbiota

262    In total, 4,038,850 paired end sequences with an average sequence length of

263    373 nucleotides (nt) were obtained. Briefly, 2,579 sequences (0.06%) were rejected

264    because they failed the SILVAngs pipeline quality control. Finally, 4,036,271

265    sequences (2334-246525 per sample) were further analyzed (Table 3 in S1). In total

266    1,129,221 (27.9%) clustered sequences with 98% sequence identity to each other,

267    including 2,665,936 (66.0%) replicates resulted to in 241,114 OTUs (5.9%). OTUs

268    were assigned to taxonomic paths (phylogenetic groups) with maximum resolution at

269    the genus level. Chloroplast, mitochondrial and archaeal 16S rRNA gene sequences

270    were detected with a relative abundance of 20.13% (190,605 sequences), 0.07%

271    (9,430 sequences), 0.02% (1,064 sequences) of the total sequences, respectively

272    (Table 3 in S1). Sequences that did not match any known taxa (sequence similarity

273    <93% to the next known taxon) were classified as "no relative" and had 0.3% relative

274    abundance (5,131 sequences) (Table 3 in S1). The sequences belonging to the

275    *Archaea*, chloroplasts, mitochondria and no relative groups were excluded from further

analysis. Only sequences assigned to the domain *Bacteria* 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 *Bacteroides* (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 3b). In samples from the interdigital region and the ear canal *Proteobacteria* were most abundant followed by *Actinobacteria*, *Firmicutes*, *Bacteroides* and *Cyanobacteria.* Groin samples were dominated by *Actinobacteria* followed *Proteobacteria*, *Firmicutes*, *Bacteroides* and *Cyanobacteria*. In the samples from the axilla *Firmicutes* was most abundant followed by *Actinobacteria*, *Proteobacteria*, *Bacteroides* and *Cyanobacteria* (Fig 3b; 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%) followed by *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 ±

14

352 7.6%) in the ear canal followed by *Clostridium sensu stricto 7*, *Sphingomonas*,

353 *Psychrobacter*, *Pelomonas*, *Flavobacterium*, *Deinococcus* and *Pseudomonas*.

354 Subsequently, each of the four taxa with the highest mean relative abundance per body

355 site (axilla: *Macroccocus;* interdigital: *Clostridium sensu stricto 7;* groin:

356 *Staphylococcus;* ear canal: *Brevibacterium*) was evaluated individually for significant

357 different abundance between the four body sites. None of these taxa had a significantly

358 different relative abundance between the four body sites (normality failed; Kruskal-

359 Wallis-test for axilla vs interdigital vs groin vs ear canal; *Macroccocus*: p=0.975;

360 *Clostridium sensu stricto 7*: p= 0.085; *Staphylococcus*: p= 0.288; *Brevibacterium*: p=

361 0.406).

362

363 **Fig 3.** Phylogenetic composition of the skin and ear canal of non-allergic versus allergic

364 dogs. (a) Relative abundance of the 10 most common bacterial phyla identified in axilla,

365 interdigital, groin and ear canal in non-allergic and allergic dogs. (b) Variation of the

366 relative abundances of the 10 most abundant phyla among non-allergic and allergic

367 dogs. Asterisks are representing statistical significance: * p < 0.05; ** p < 0.01; *** p <

368 0.001.

369

370 **Changes in the Skin Microbiome of allergic German**

371 **shepherd dogs**

372 **Diversity and richness of skin microbiota of allergic versus non-**

373 **allergic GSDs**

374 A significantly lower bacterial community richness was determined at the skin of the

375 axilla of allergic GSDs (A; Chao 1 index; p= 0.032; Fig 5a). This finding was supported

376 by respective rarefaction curves showing that the samples from the axilla of non-

377 allergic dogs had a higher slope than the curves from the samples of allergic dogs

378 indicating the presence of more complex bacterial communities in non-allergic dogs

379 (Fig 6). The evenness of the skin microbiota in the groin of allergic dogs was

380 significantly lower because of the high abundance of individual phylogenetic groups

381 (Evenness index values, Table 9 in S1; Fig 4 in S2).

382

383 **Fig 4.** Box-plots of alpha diversity index Shannon (total diversity) of the skin microbiota

384 per body site (axilla: (a); interdigital (b); groin (c); ear canal (d)) of non-allergic and

385 allergic German shepherd dogs, based on Illumina 16S rRNA gene amplicon

386 sequencing of microbial communities.

387 **Fig 5.** Box-plots of alpha diversity index (number of taxa considering the number of

388 singletons) per body site (axilla: (a); interdigital (b); groin (c); ear canal (d)) of non-

389 allergic and allergic German shepherd dogs using Chao 1 index, based on Illumina

390 16S rRNA gene amplicon sequencing of microbial communities. Asterisks are

391 representing statistical significance: * $p < 0.05$.

392 **Fig 6.** Rarefaction curves (specimens versus taxa) based on Illumina 16S rRNA gene

393 amplicon sequencing of microbial communities from the axilla (A) of allergic (a-) and

394 non-allergic GSD. The sample sites are arranged as the curves in descending order

395 and with an equivalent color.

396

397 **Differences in the composition of bacterial communities of allergic**

398 **versus non-allergic GSDs**

399 Shifts in the bacterial community composition at the skin of allergic dogs were

400 visualized in NMDS plots (Fig 7). Significant differences were obtained for the axilla

401 (A; One way ANOSIM p=0.048, R=0.1001; One way PERMANOVA p=0.0256), groin

402 (L; One way ANOSIM p=0.036, R=0.00386; One way PERMANOVA p= 0.0264) and

403    ear canal (O; One way ANOSIM p=0.0012, R=0.1842; One way PERMANOVA p=

404    0.0025), but not for the interdigital area (Int; One way ANOSIM p=0.1548; One way

405    PERMANOVA p= 0.1171). The inclusion of the health status (non-allergic vs allergic)

406    confirmed that allergy correlated with the significant differences of the clustering of the

407    community patterns between the samples (Fig 7). Sample a18O (ear canal of the

408    allergic dog a18) was excluded from the NMDS plot because the community was too

409    different (Fig 5 in S2). The strong difference of the community profile was due to the

410    high relative abundance of the genus *Staphylococcus* (91.3%) compared to 0.02-6.4%

411    in the other samples (Table 6 in S1).

412        In order to determine which taxa (phylogenetic groups/genera) had the main

413    contribution to the community differences among allergic versus non-allergic GSD for

414    each body site, SIMPER analysis was performed. Phylogenetic groups with the highest

415    contributions (>2%) are listed in S1 Table 10. The genus *Macrococcus* (9.0%

416    contribution) was the major contributor for the differences in the samples from the axilla

417    (A), followed by *Staphylococcus* (4.7% contribution). For the samples from the groin

418    (L) the genus *Staphylococcus* contributed the most (7.0% contribution). For the ear

419    canal (O), *Brevibacterium* contributed the most to the differences (2.5% contribution)

420    with S*taphylococcus* of the sample a18O being excluded as dominated all the other,

421    as mentioned above, affecting the comparison.

422        In order to evaluate if oclacitinib had an effect in the composition of the skin

423    microbiota of the allergic dogs, we compared the bacterial communities' composition,

424    as described above for the subgroups allergic with oclacitinib (n=6) and without

425    oclacitinib (n=6). No significant difference was obtained, proposing that oclacitinib did

426    not affected the cutaneous and ear canal microbiota composition (One way ANOSIM

427    p-values; axilla: p= 0.2777; interdigital: p= 0.2949; groin: p=0.1494; ear canal: p=

428    0.9022) among the allergic dogs, given the small number of the subgroup population.

429  On this basis we assume that apoquel did not affect the comparison between allergic

430  and non-allergic dogs.

431

432  **Fig 7.** Comparative analysis of the skin microbiota composition of relative abundance

433  patterns at each body site of non-allergic (black color) and allergic (red color) dogs,

434  performed by NMDS analysis based on a Bray-Curtis similarity matrix. The health

435  status was included as environmental parameter; (A- Axilla, Int- interdigital area, L-

436  groin, O- ear canal)

437

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

440      The order of the 10 most abundant phyla of allergic GSDs was different

441  compared with non-allergic GSDs (Table 5 and Table 11 in S1). At the skin of allergic

442  GSDs *Proteobacteria* (mean relative abundance 35.5 ±10.9%) and *Actinobacteria*

443  (22.4 ±10.2%) were the most abundant phyla dominating in each of the four sample

444  sites, followed by *Firmicutes* (17.0 ±14.6%) and *Bacteroidetes* (14.1 ±5.2%) (Figs 3a

445  and 3b; Table 11 in S1). Between the two groups, *Proteobacteria* (p< 0.001) and

446  *Bacteroidetes* (p= 0.003) had a significantly higher mean relative abundance at the

447  skin of allergic dogs, whereas *Actinobacteria* (p= 0.012), *Deinococcus-Thermus* (p<

448  0.001) and *Verrucomicrobia (*p= 0.016) occurred in a significantly lower relative

449  abundance in allergic dogs. *Firmicutes* showed no significant differences between

450  allergic and non-allergic GSDs (Fig 3b; Table 11 in S1).

451      *Sphingomonas* and *Staphylococcus* dominated the skin microbiota of allergic

452  GSDs, followed by *Clostridium sensu stricto 7, Nocardioides* and others (Tables 8a

453  and 8b in S1; Fig 8). Evaluating the mean relative abundances of the phylogenetic

454  groups for significant differences between allergic and non-allergic GSDs showed that

455    *Sphingomonas* was significantly higher abundant in the allergic GSDs (p<0.001; mean

456    rel. abundance of non-allergic 2.5 ±1.4% versus 4.9 ±2.3% of allergic dogs), as well as

457    *Nocardioides* (p=0.034; mean rel. abundance of 2.5 ±1.8% for non-allergic dogs versus

458    4.3 ±3.4% for allergic dogs). No significant difference was observed for

459    *Staphylococcus* (p=0.8, Table 7) and *Clostridium sensu stricto 7* (p=0.062) between

460    allergic and non-allergic GSDs. Interestingly, allergic dogs had significantly lower mean

461    relative abundance of *Macrococcus* (p<0.001; mean rel. abundance of 4.8 ±15.5% for

462    non- allergic dogs versus 0.1 ±0.4% for allergic dogs; Table 9 in S1) and

463    *Brevibacterium* (p= 0.016; mean rel. abundance of 2.7 ±6.3% for non- allergic dogs

464    versus 0.1 ±0.3% for allergic dogs) than non-allergic GSDs.

465        A more detailed comparison between the allergic and non-allergic GSDs of the

466    most abundant taxa per body site revealed important findings. *Clostridium sensu stricto*

467    *7* occurred in a significantly higher relative abundance on the axilla of allergic dogs (p=

468    0.026; mean relative abundance of 2.7 ±5.0% for non-allergic dogs versus 6.2 ±7.8%

469    for allergic dogs). *Nocardioides* of the interdigital skin showed no significant difference

470    (p= 0.138). *Sphingomonas* was significantly more abundant in the groin of the allergic

471    dogs (p= 0.002; mean relative abundance of 2.0 ±1.5% for non-allergic dogs versus

472    4.9 ±2.4% for allergic dogs). In addition, *Sphingomonas* was significantly more

473    abundant in the allergic versus non-allergic GSDs for multiple sites (axilla: p= 0.013;

474    Interdigital: p= 0.014; ear canal: p= 0.017; Fig 8; Table 8 in S1). *Staphylococcus* did

475    not show any significant difference for any site between the allergic and non-allergic

476    GSDs (Table 12a in S1). On the contrary, a significantly lower relative abundance of

477    *Macrococcus* was obtained from samples of the interdigital skin, the groin and the ear

478    canal of the allergic dogs but there was no significant difference for the axilla (Table

479    12b in S1). In addition, *Brevibacterium* was also evaluated for significant difference for

480    the ear canal samples because it was the main contributor to the difference of the

481   bacterial community composition between the two groups in SIMPER analysis, with

482   the allergic group having a significantly lower mean relative abundance (p= 0.041;

483   mean relative abundance of 3.6 ±7.7% for the non-allergic dogs versus 0.1 ±0.4% for

484   the allergic dogs).

485

486   **Fig 8.** Relative abundance of the 10 most abundant taxa of non-allergic and allergic

487   dogs. Asterisks are representing statistical significance: * p< 0.05; ** p< 0.01; *** p<

488   0.001.

489

## Discussion

491   In this NGS study, we described the bacterial community of the skin and ear

492   canal of non-allergic GSDs and compared it to the skin and ear canal microbiota of

493   allergic GSDs. Several NGS studies have also examined the canine skin or ear canal

494   in health and disease. These studies used different techniques, from sample collection

495   and storage, to DNA extraction and analysis and as shown before, methodology

496   influences results [42–44]. Therefore, a direct side by side comparison of studies is

497   difficult [42]. Nevertheless, any of those results is useful to further characterize the

498   canine cutaneous microbiota in health and disease. Our 16S rRNA gene amplicon

499   approach (nested PCR; V3-V6 & V4-V5) showed high individual and body site

500   variability between the different study subjects, without significant differences between

501   the body sites, in contrast to the first NGS-based study in veterinary medicine [13]. In

502   our study the body skin (axilla, interdigital, groin) showed significantly higher species

503   richness than the ear canal in non-allergic dogs. Possible explanations may include

504   the special ear canal construction (chamber-like) and histology, with a comparatively

505   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 [45,46]. Bactericidal activity of cerumen could explain the lower species richness compared to the other body sites. The influence of pH on species richness was not assessed in this study. There are few studies evaluating the cutaneous pH in dogs in health and disease. The canine cutaneous pH is considered relatively more alkaline than the human one with ranges from 4.84 to 9.95 [47]. The properties and functions of cutaneous pH have been reviewed and *in vitro* as well as *in vivo* studies have shown that cutaneous pH can affect the growth of skin bacteria but the extent of the effect on skin microbiota has not been fully determined [47]. Nevertheless, it has recently been shown that canine cutaneous pH has a positive correlation with alpha diversity metrics of the cutaneous microbiota [14]. Therefore, we want to emphasize the need for measurement of pH in future NGS cutaneous microbiota studies.

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. Furthermore, studies have shown that environmental factors can influence the skin microbiome in humans like contact with soil or plant material [48–51]. Therefore, it can be assumed that different habits or environment of the dogs could explain differences in results. Both, living in the same household and sex affected the bacterial communities patterns among the samples of the healthy dogs, in agreement with other studies [48,52]. 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

532 and the axilla of the GSDs. One possible explanation for the interdigital area could be

533 direct and repeated contact with soil, where it is shown that Proteobacteria

534 predominate its bacterial composition [53]. Currently there are only two other NGS

535 studies describing the canine ear canal. In the first study, Proteobacteria were the most

536 abundant phyla followed by Actinobacteria, Firmicutes and Bacteroidetes with

537 Fusobacteria and *Escherichia* as the most abundant genera [1]. The second study

538 showed also similar most abundant phyla but in a different order, with Firmicutes being

539 most abundant followed by Proteobacteria, Bacteroidetes and Actinobacteria with

540 *Romboutsia* as the most abundant genus [54]. Our findings were consistent with the

541 first study [1], as we had the same order of the most common phyla, except that we

542 documented Cyanobacteria instead of Fusobacteria. At the genus level we identified

543 *Brevibacterium* as the most abundant taxon. The most abundant genus found on non-

544 allergic skin was *Macrococcus* with the highest abundance in the axilla. The second

545 most common genus was *Staphylococcus* with the highest abundance in the groin

546 followed by the axilla, the interdigital area and the ear canal. *Clostridium sensu stricto*

547 *7* was the most common genus of the interdigital area. In contrast, one previous study

548 found that healthy canine skin was predominantly colonized by *Porphyromonas*,

549 *Staphylococcus*, *Streptococcus* and *Propionibacterium*, with *Porphyromonas*

550 significantly colonizing the axilla [14]. In another study, *Ralstonia* was the most

551 abundant genus (lip commissure and conjunctiva) [13]. A recent exploratory study with

552 six healthy dogs showed that on the skin (inguinal, axilla, periocular and trunk),

553 *Pseudomonas* was most abundant followed by *Kocuria*, *Porphyromonas* and

554 *Corynebacterium* [55], while another one identified *Propionibacterium acnes*,

555 *Corynebacterium* and *Porphyromonas* as most abundant [48]. These findings are in

556 contrast to our study. *Macrococcus* is a Gram-positive coccoid bacterium, previously

557 placed into the *Staphylococcus* genus but since 1998 assigned to its own genus [56].

It is composed of eight species that are closely related with species of the genus *Staphylococcus* [56]. 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 [56]. Even though it is considered as a non-pathogenic bacterium, there are a few reports of infections associated with *Macrococcus caseolyticus* and *M. canis* [57–60]. 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 [57]. As both healthy and infected skin was colonized, it was concluded that *Macrococcus* is an important opportunistic bacterium of the canine skin [57]. Our study underlines this, as *Macrococcus* contributed mainly to the difference in bacterial community composition of the axilla between non-allergic and allergic GSDs. These findings highlight the need for further studies of the distribution of this bacterium in healthy and diseased dogs.

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 the operational taxonomic units (OTUs) that most contribute to Bray-Curtis measures of similarity 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

584 different bacterial communities and reduced diversity in the allergic dogs [14]. The first

585 NGS study using allergic dogs without flares, like our study population, did not show

586 significant differences of the skin microbiota community composition in comparison

587 with healthy dogs in contrast to our findings, but did show that allergic skin had lower

588 diversity (richness), similar to our findings [13]. The ear canal of allergic dogs without

589 signs of otitis also had a bacterial composition significantly different from healthy dogs'

590 ear canal, and without a significant difference in diversity between the groups, similar

591 to our results [1]. A trend of dysbiosis in association with allergy was shown with

592 significantly increased abundance of *Staphylococcus* and *Ralstonia* in the atopic ear

593 canals [1]. In general, we were able to show a trend of dysbiosis for all the sites

594 evaluated in total, with significantly reduction of *Macrococcus* and *Brevibacterium* and

595 increase of the Phylum Proteobacteria and the genera of *Sphingomonas* and

596 *Nocardioides*.

597       Six out of 12 allergic dogs received oclacitinib, which is a Janus kinase inhibitor

598 with anti-pruritic and anti-inflammatory properties [61]. Until now there is no study

599 investigating the impact of oclacitinib on the skin microbiota. One study documented

600 that treatment with cyclosporine or corticosteroids did not affect the cutaneous

601 microbiota in dogs, evaluating these dogs before, during and after treatment [62]. This

602 is in line with our findings, bacterial composition of allergic dogs with and without

603 oclacitinib did not show any significant differences. Thus, we suggest that oclacitinib

604 may not influence the overall comparison between the non-allergic and allergic dogs,

605 but future studies with larger population should confirm our finding.

606       One limitation of our study was the small sample size. However our sample size

607 is larger than in previous studies [1,13,55,62]. Furthermore, our study adds to a

608 growing corpus of research that helps us better understand the microbiota inhabiting

609 the skin and ear canal of dogs and can be used to design larger confirmatory studies.

610 The study was a cross-sectional analysis and therefore it remains unclear if findings

611 are a cause or a result of allergy. Regarding the description of the cutaneous

612 microbiota only a longitudinal study could clarify if the composition of the microbiota is

613 stable or only transient. Further studies including also non-allergic dogs from other

614 breeds are required, in order to evaluate if the skin microbiota of non-allergic GSDs is

615 breed specific or not. Because previous studies of humans' skin microbiota detected a

616 low amount of microbial DNA when standard PCR was used, a nested PCR was

617 chosen in our study [24,25]. A bias in alpha diversity and community structure has

618 been documented due to a nested PCR in stool samples but not in vaginal swabs and

619 might be considered as a possible limitation factor of our study [63]. Two allergic dogs

620 with mild skin lesions (CADESI-4 score: 2 and 4) had a PVAS score of 8/10 and 7/10,

621 possibly due to an initiating flare state. In order to minimize the possibility to include a

622 dog with a subclinical cAD into the non-allergic group, the age of the healthy dogs had

623 to be more than four years old. Consequently, the age of dogs of two groups was

624 significantly different. This might be a limitation, as it has been shown in humans that

625 the skin microbiota evolve with age [64–66]. In addition, all dogs were living in the same

626 area of Germany and the environment affects skin microbiota [67], possibly leading to

627 differences between results of studies from other counties. In our study, we did not

628 evaluate the association of rural or urban environment with the dogs' cutaneous

629 microbiota. A last limitation of our study is that the 16S rRNA gene sequencing cannot

630 differentiate dead from alive bacteria [2]. Larger studies including other breeds, several

631 countries, as well as dogs with and without allergy flares could elucidate the canine

632 skin microbiota in health and disease.

## 633 Conclusion

634    This is the first study describing and comparing the cutaneous and ear canal

635    microbiota of non-allergic and allergic GSDs, showing that allergic dogs have a

636    significantly different bacterial community composition and significantly lower species

637    richness (alpha diversity). The skin and ear canal microbiota of non-allergic GSDs is

638    highly variable, and the ear canal has a significantly lower species richness than the

639    body skin.

640

641    **Acknowledgment**

644

645    **Conflict of interest:**

646    Non declared

647

648    **Supporting information**

649    **S1 Tables 1 - 12**

650    **S2 Figs 1 - 5**

651

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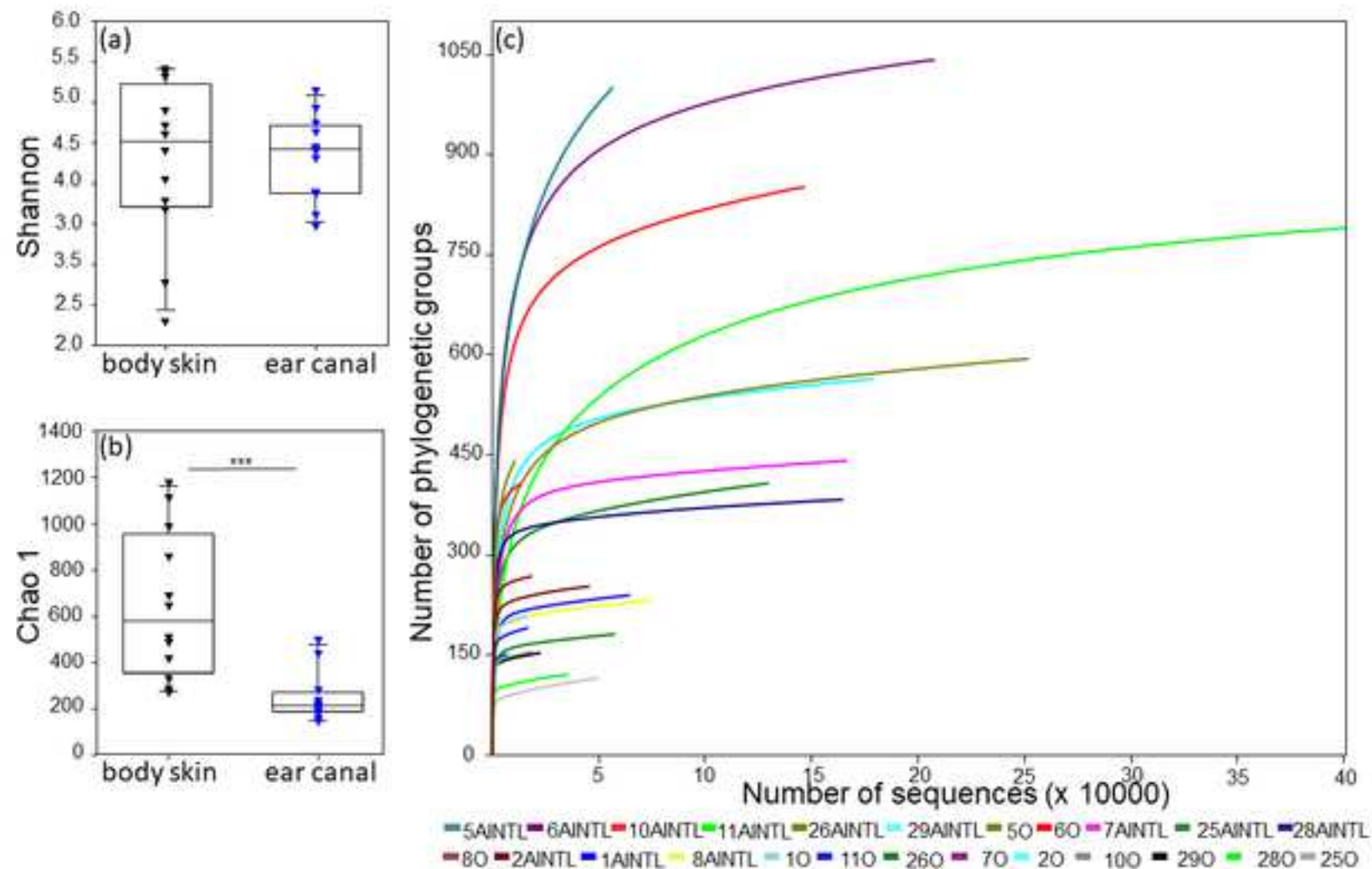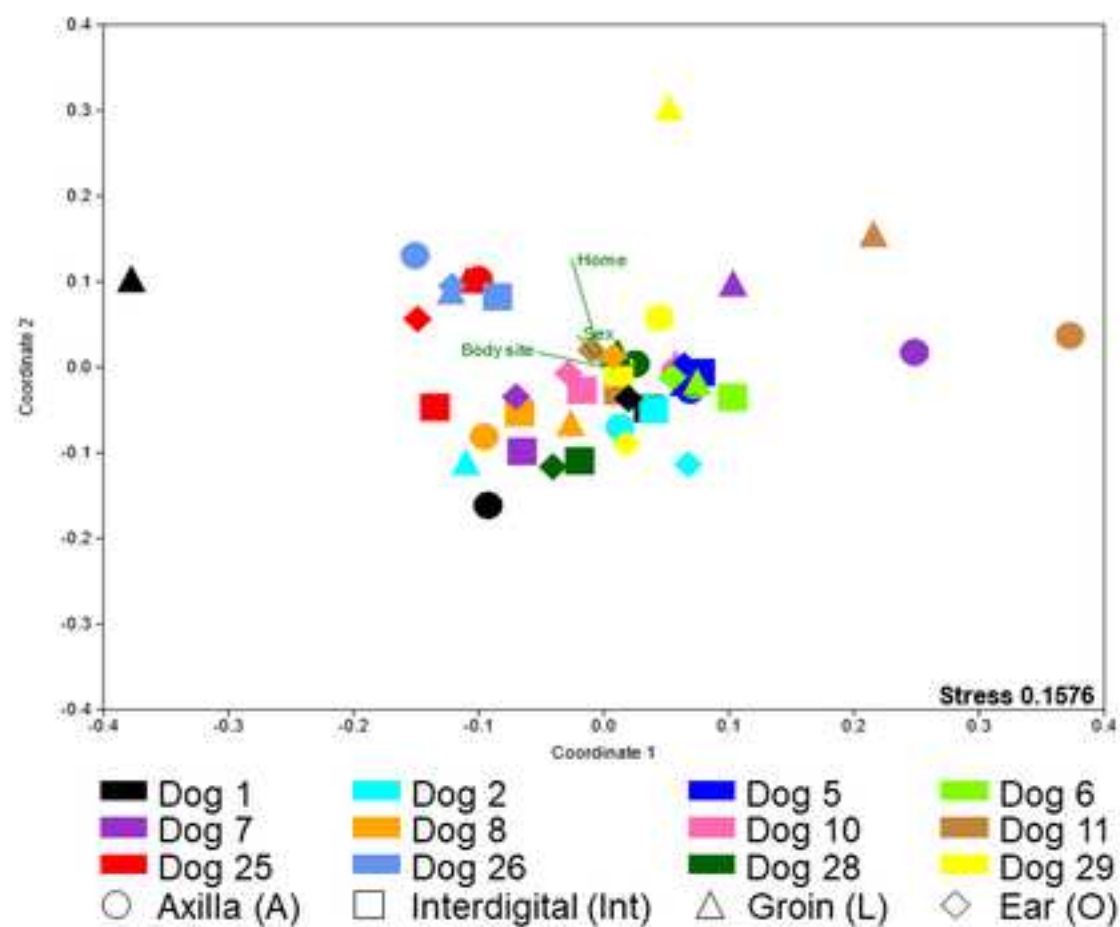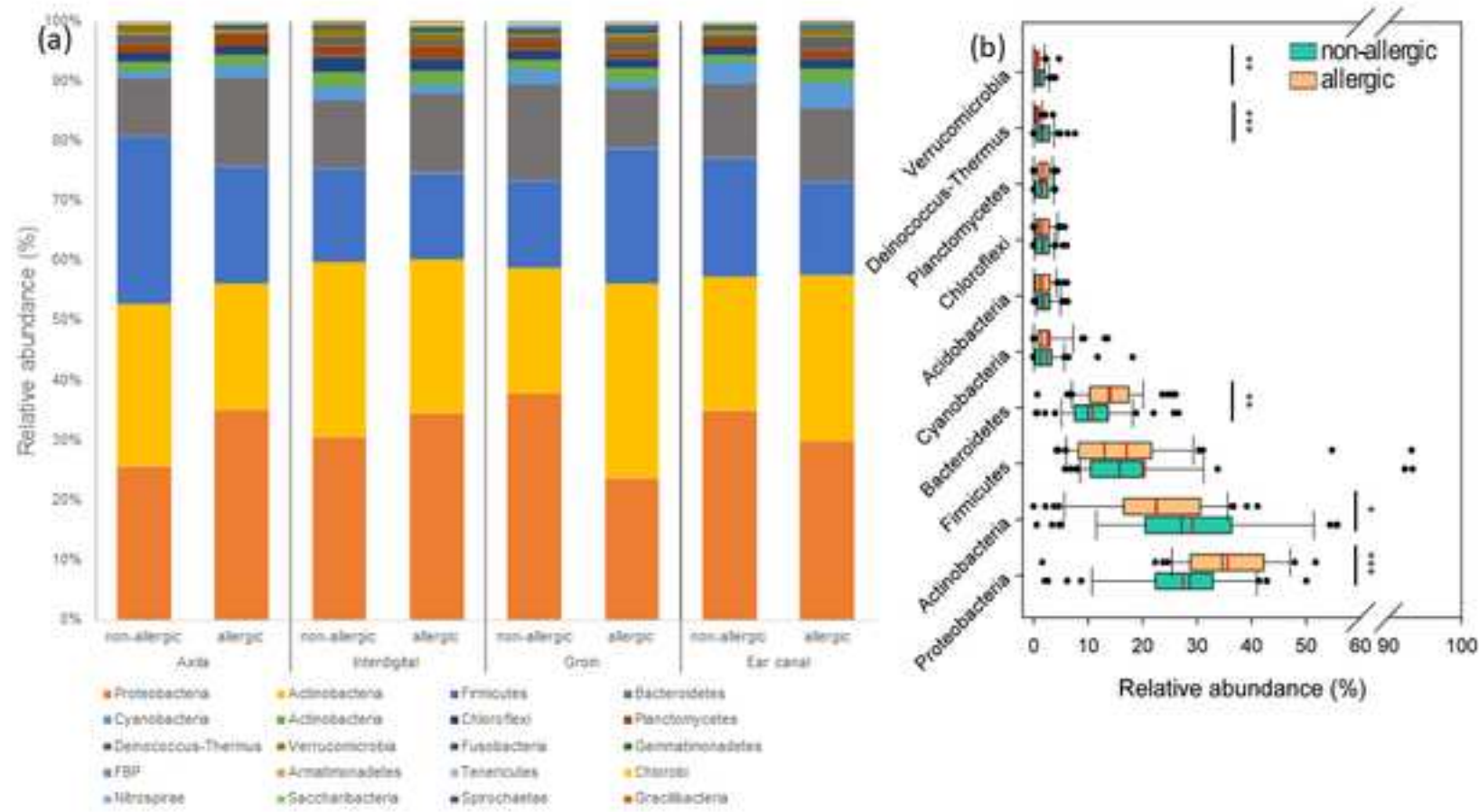866     doi: 10.1073/pnas.1719785115.

Figure 1

Figure 2

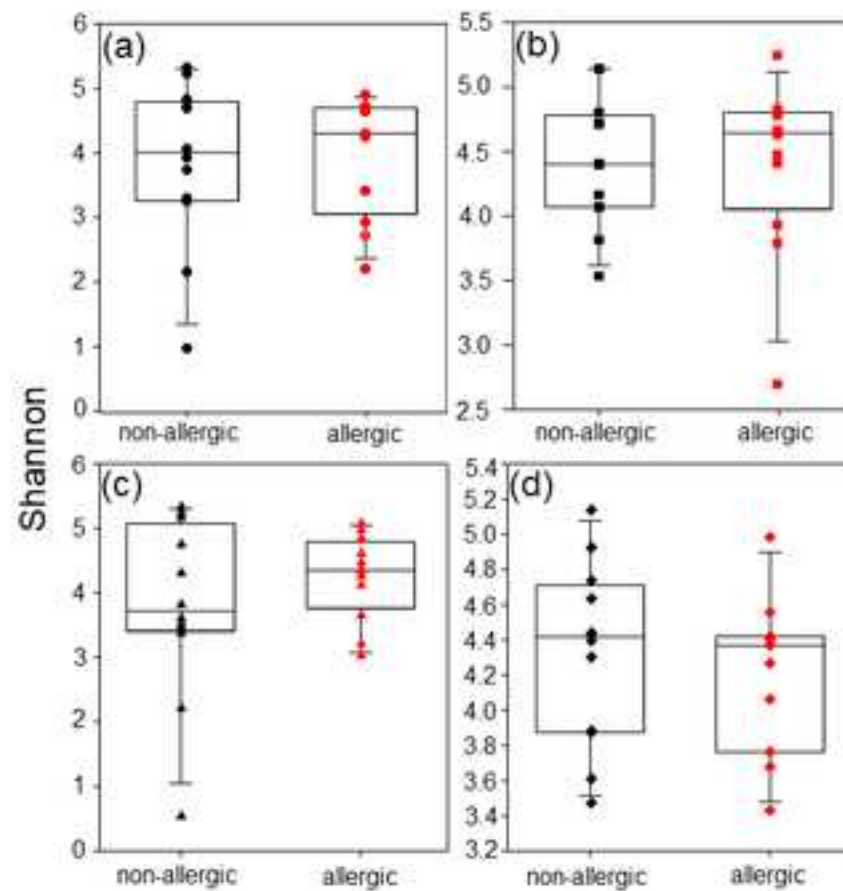Figure 3                                                                                    Click here to access/download;Figure;Fig3.tif.TIF ⬇

Figure 4

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Figure 5

Figure 6

Figure 6

Figure 7

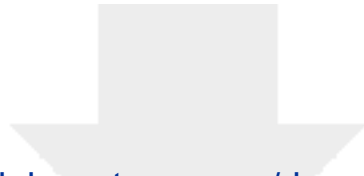Click here to access/download;Figure;Fig7.tif.TIF
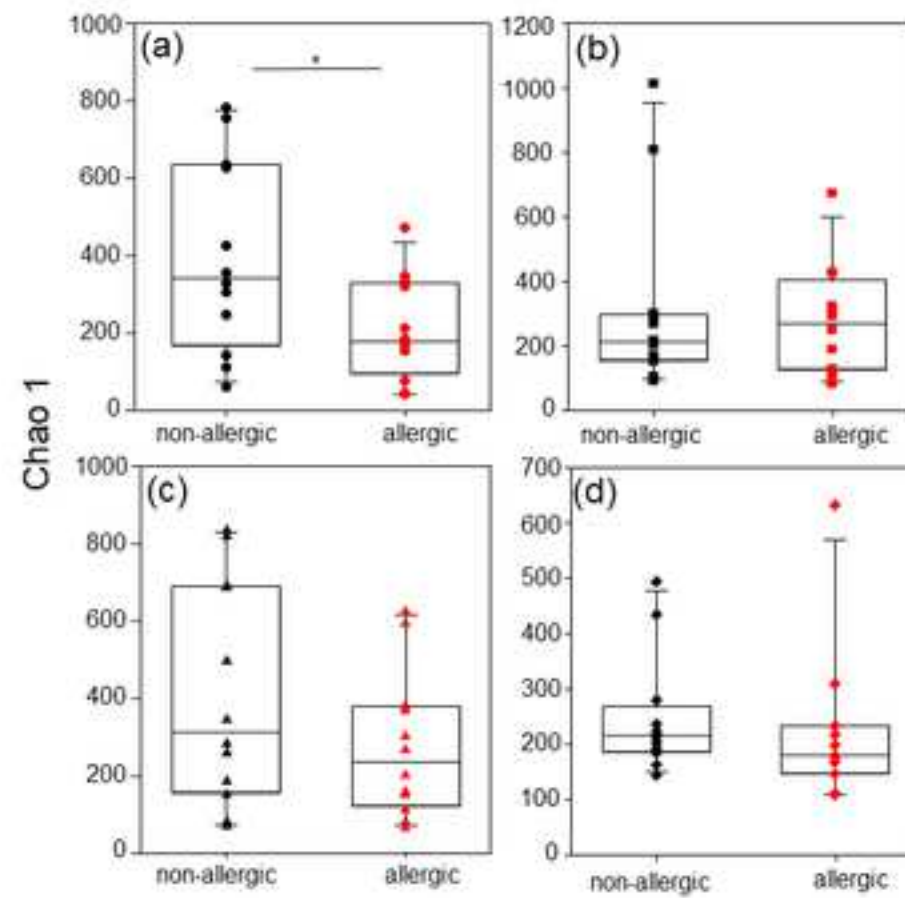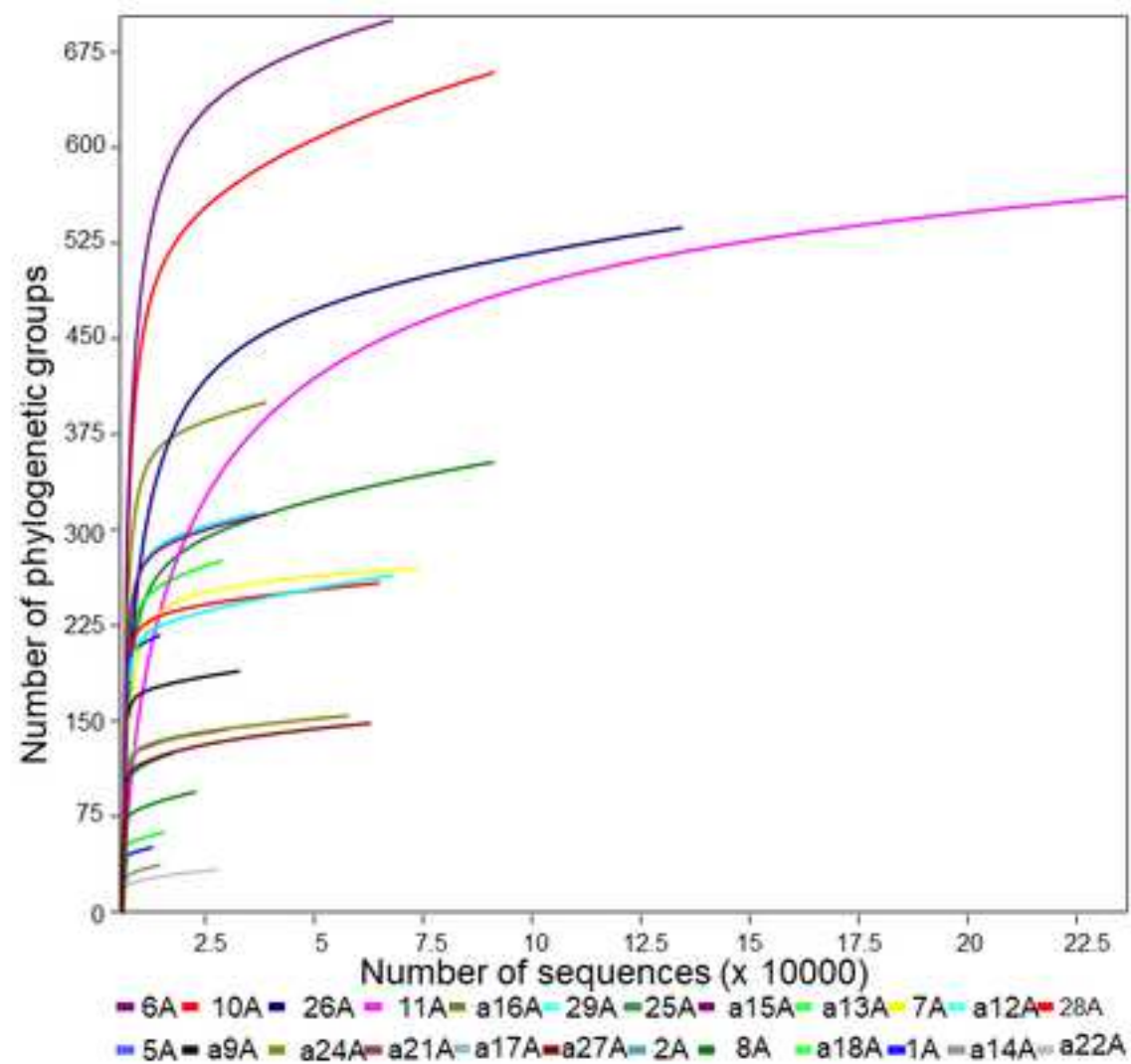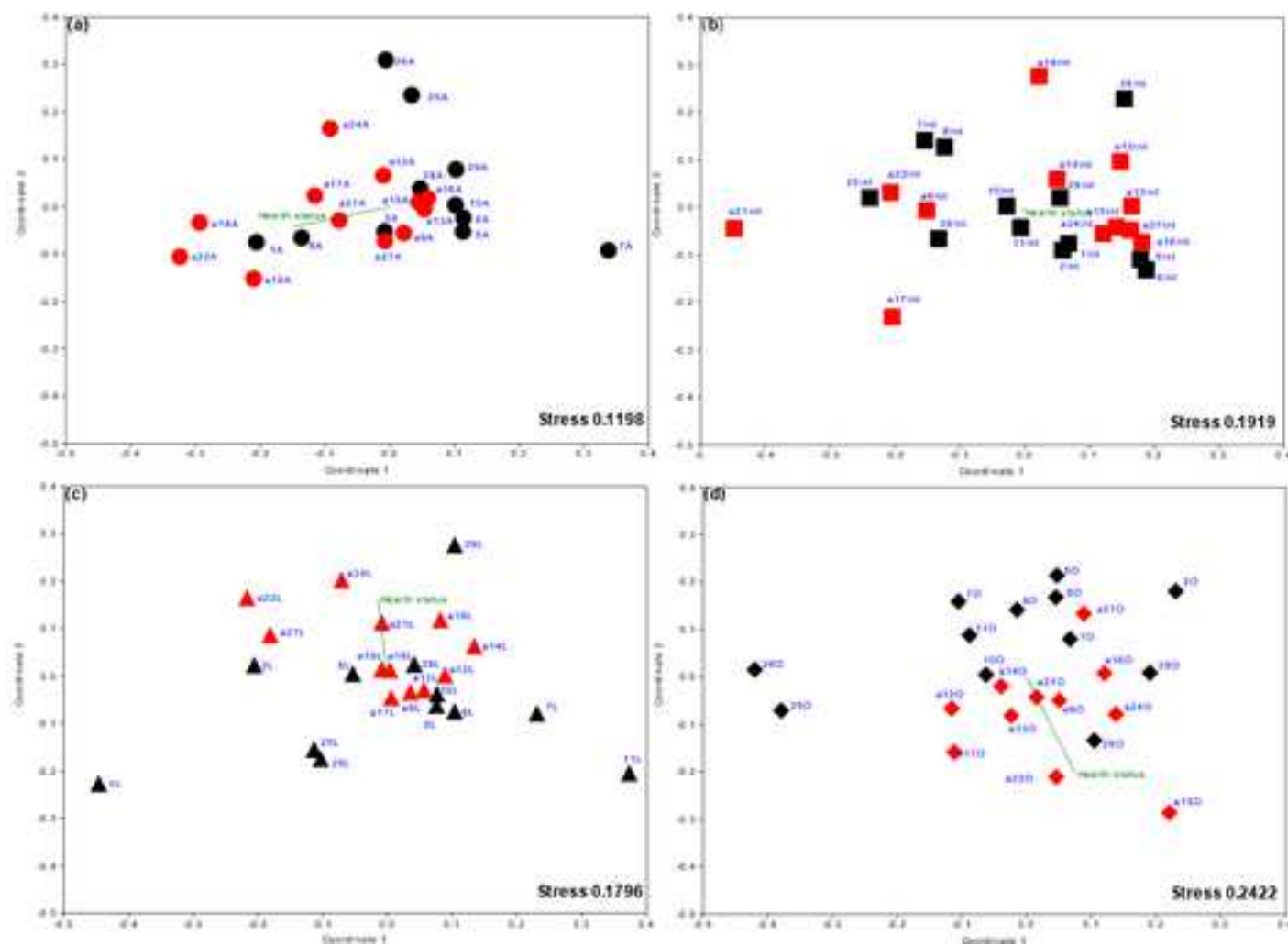
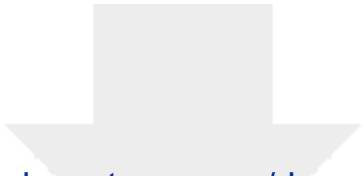Figure 8

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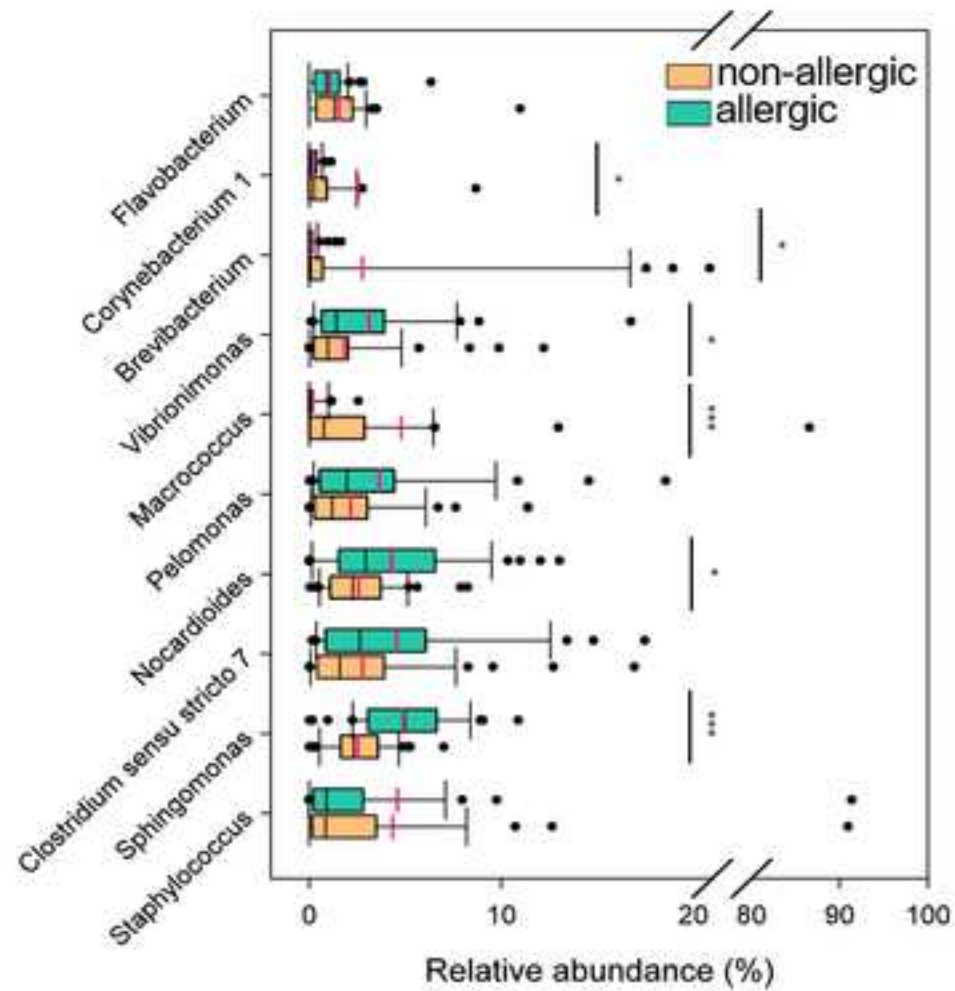
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